Male Gametophyte Development and Evolution in Extant Gymnosperms

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ABSTRACT

The male gametophytes of gymnosperms are characterized by the diversities in pollen morphology, cellular composition and pattern of cell division, pollen tube morphology, sperm delivery, growth pattern through the ovule and nucellus, and pollen tube wall composition both within and among the four living orders, i.e., Cycadales, Ginkgoales, Coniferales, and Gnetales. At dehiscence, gymnosperm pollen grains contain a variable number of cells yet none have sperm at this stage. Pollen germination in the ovule usually occurs within a few hours or days in gnetophytes, about a week or so in conifers and Ginkgo, or after several months in cycads. Complete development of the male gametophyte typically involves two to five mitotic divisions. Evolution of the male gametophyte appears to have involved a reduction of its component cells with prothallial cells being among those reduced or eliminated. There is a shift in the site of sperm discharge from a proximal position in pollen grains of cycads and Ginkgo to distal in conifers and gnetophytes. Two methods of sperm delivery occur in gymnosperms: zooidogamy, defined by pollen tubes with motile sperm as exhibited in cycads and Ginkgo, and siphonogamy, defined by pollen tubes with non-motile sperm which are directly delivered into the egg as exhibited in conifers and gnetophytes. Different pollen tube morphologies occur in the nucellus, i.e., branched and haustorial in cycads and Ginkgo, and unbranched and non-haustorial in conifers and gnetophytes. Pollen tubes form heterotrophic relationships with the nucellus, but it is only in cycads that intracellular penetration results in significant destruction of the nucellus. Pollen tube walls of gymnosperms contain cellulose and arabinogalactan proteins; however, pectins are prevalent in cycads and mixed β-glucan in Ginkgo. A standard terminology to describe the cellular composition of the male gametophytes in gymnosperms is proposed.

Keywords: pollen grain, pollen tube, siphonogamy, sperm, sexual reproduction, zooidogamy

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INTRODUCTION

The appearance of pollen grains is a key innovation in seed plant evolution. Before pollen grains, fertilization was restricted to a wet environment that was required for the sperm to swim to the archegonia containing the egg cell. This mode of fertilization still occurs in land plants such as bryophytes, lycophytes and pteridophytes. This innovation of the male gametophyte increased the success of fertilization in seed plants by eliminating the chances and hazards associated with the aquatic transfer of sperm (Gifford and Foster 1989). Within the gymnosperms, and seed plants in general, two methods of sperm delivery occur: zooidogamy and siphonogamy. In zoogamous seed plants such as the cycads and Ginkgo, the pollen tube function as haustoria, i.e., multiple branches emerge at their distal end that penetrate the nucellus to absorb water and nutrients for the development of the sperm. At their proximal end, the pollen tube releases flagellated sperm into a specialized chamber in the ovule from which the sperm subsequently swim to the egg cell (Friedman 1993; Poort et al. 1996; Rudall and Bateman 2007). The fossil record also provides several examples of zooidogamy in extinct gymnosperms (Poort et al. 1996; Nishida et al. 2003, 2004; Leslie 2008). The discovery of the zoogamous mode of fertilization in Ginkgo biloba (Hirase 1896) and Cycas revoluta (Ikeno 1896) linked gymnosperms with pteridophytes, which is considered as one of the seminal biological discoveries in the 19th century. In conifers and gnetophytes, pollen tubes elongate from the nucellus to an archegonium (or female gametophyte in Gnetum and Welwitschia) and deliver nonflagellated sperm directly into the egg (siphonogamy). The shift in the posi-
tion of sperm release and loss of sperm locomotion are among the key traits in the transition towards siphonogamy (Poort et al. 1996; Rudall and Bateman 2007), the predominant mode of fertilization in seed plants. Phylogenetic analyses show that siphonogamy may have evolved at least twice in gymnosperms, once within the conifers and once in the gnetophytes (Doyle and Donoghue 1992; Friedman 1993; Rudall and Batemen 2007). Siphonogamy occurs in both extant gymnosperms and angiosperms (two extant genera) and sporophyte concentration is considered different (Gifford and Foster 1989; Fernando et al. 2005a). There is also a marked difference in the rates of pollen tube growth and fertilization intervals between gymnosperms and angiosperms (Williams 2008).

The process of male gamete formation has been reviewed for higher plants, but has focused on angiosperms, with no inclusion of gymnosperms (see Boavida et al. 2005). Where gymnosperms were included, as reviewed by Moitra and Bhatnagar (1982), description of the male gametophyte concentrated on pollen grain and gamete development, with minor mention of pollen tube development. A comparison of the male gametophytes among seed plants has been carried out but focused on the evolution of pollen grain polarity and siphonogamy (see Rudall and Batemen 2007). In gymnosperms, a review of pollen tube growth and development is available (see Fernando et al. 2005a), but it is limited to the conifers. Therefore, further discussion is needed on the nature of pollen grains and tubes in the extant gymnosperms.

This review will describe male gametophyte development focusing on morphology, polarity and cellular composition of the pollen grain, pollen germination and tube growth, sperm formation, growth of the pollen tubes in the ovules, chemical composition of the intine and pollen tube wall, and evolution in the four living orders of gymnosperms: Cycadales, Ginkgoales, Coniferales, and Gnetales. A standard terminology to describe the cellular composition of male gametophytes in gymnosperms, which is consistent with that of the angiosperms, is proposed. Immature or developing male gametophyte refers to all stages starting from the formation of the microspores, except sperm formation. Formation of the sperm indicates that the male gametophyte is mature. The concept of the nature of sperm in gymnosperms is confusing, i.e., whether they are composed of cells or nuclei. Therefore, this review will clarify which extant gymnosperms have sperm cells and which have sperm nuclei. By studying the development and evolution of the male gametophytes of the living gymnosperms, this review will help further our knowledge of the reproductive processes in the more primitive seed plants and increase our awareness of the sexual reproductive diversity in seed plants.

**EVOLUTION AND CLASSIFICATION OF GYMNOSPERMS**

Stratigraphic evidence places the origin of Coniferales in the Upper Carboniferous (Rothwell et al. 1997; Hernandez-Castillo et al. 2009). Cycadales (Mamay 1976; Gao and Thomas 1989) and Ginkgoales (Rothwell and Holt 1997; Royer et al. 2003) in the Permian, and Gnetales in the Triassic (Stewart and Rothwell 1993; Crane 1996; Rydin et al. 2006). From Permian to Late Jurassic, many seed plants became extinct including lycophytes, medullosans, Callophyllaceae, glossopterids, Cordaitales, and Voltziales (Stewart and Rothwell 1993). The Cycadales reached their greatest abundance during the Jurassic then began to decline at the end of the Mesozoic era; however, a few families endured to give rise to the modern cycads (Mamay 1969, Gao and Thomas 1989). Similarly, the Ginkgoales reached their greatest abundance and widest distribution during the Jurassic; however, before the end of the Cretaceous, the order diminished and is now represented by a single genus, Ginkgo (Rothwell and Holt 1997; Royer et al. 2003; Zhou and Zheng 2003). The Coniferales began extensive diversification during the Mesozoic and maintains moderate diversity to this day. In spite of the extinction of many of its members, conifers are still a dominant group based on number of extant species (627 species according to Farjon 2008), number of individuals, and distribution.

Modern cycads occupy a remnant of their former range and are composed of three families, eleven genera, and about 300 species: Cycadaceae (one genus), Stangeriaceae (two genera), and Zamiaceae (eight genera) (Norstog and Nicholls 1997; Hill et al. 2003, 2005). These genera are sharply defined and, although all exist in tropical or subtropical areas, there is no genus found in both the Eastern and Western hemispheres. In addition, most cycads are local in distribution, except for Zamia, which extends from Florida to Chile, and Cycas, from Japan to Australia, but none form continuous natural stands (Hill et al. 2005). Cycads are characterized by sturdy, branched or unbranched trunks covered with persistent leaf bases and topped with a crown of large, pinnately compound leaves. All species are dioecious, i.e., individual plants bear either separate male or female sporophylls. Cycad micro- and megasporophylls are considered to be much-reduced leaves that are usually aggregated into large, laterally displaced cones (strobili). In the case of Cycas, the basal genus, megasporophylls are loosely arranged and not clustered into cones. At the time of pollination, pollen grains are released from the male (pollen or microsporangiate) cones and transferred to the female (seed or megasporangiate) cones by wind or insects (Choi and Friedman 1991).

Although widely distributed during the Mesozoic era, the Ginkgoales was never a large order and continued as a single line into the present, represented only by a single living species, Ginkgo biloba (Coulter 1909). Recognized by its tall stature and spreading branches with bilobed, fan-shaped and dichotomously veined leaves, Ginkgo is now presumed to be extinct in the wild, but has been distributed worldwide through cultivation (Gifford and Foster 1989; Rothwell and Holt 1997; Royer et al. 2003; Shen et al. 2005). Ginkgo is dioecious, ovules are typically borne in pairs on the end of short stalks, microsporangia occur in cones, and pollen grains are wind dispersed (Chamberlain 1935).

With a fossil record extending back to the Upper Carboniferous (Pennsylvanian), conifers are considered to have arisen from primitive gymnosperms with seed-bearing cupules (Miller 1977; Meyen 1984, 1997). Single or multi-seeded, the cupules were composed of modified branchlets that fused to eventually form cone-like structures containing ovules that resulted in naked seeds. The Podocarpaceae, found in the tropics and southern hemisphere, is the first conifer family recognized in the fossil record, although most of the other families are apparent at the end of the Triassic and beginning of the Jurassic (Miller 1977, 1999; Farjon 1998). The earliest fossil evidence for Pinaceae is from the Late Jurassic (Miller 1977; Farjon 1998). During these warm periods, conifers extended towards the polar regions and higher elevations. In contrast, as temperatures cooled, conifer distribution contracted to more tropical regions. These trends may have resulted in many monotypic families, genera, and isolated species (Farjon 1998).

Early conifer evolution enabled separate and distinctive compound female cones and simple male cones to appear and remain as distinct conifer traits. Evolutionary diversification of the conifers has led to a great diversity in cone morphology, male and female gametophyte development, gamete structure, fertilization, embroyogenesis, and seed development (Singh 1978; Bruns and Owens 2000). This is particularly true of the male gametophyte and conifer pollination mechanisms (Singh 1978; Tomlinson 1994; Owens et al. 1998; Fernando et al. 2005a). Most conifers are monoecious, but all are wind pollinated; however, the pollen grain and sperm structures, the method by which pollen grains enter the ovules, the number of cells within the shed pollen grain, and the time and method by which pollen...
tubes form have evolved along several different lineages (Owens and Bruns 2000; Fernando et al. 2005a).

Conifers are the largest and most diverse of all extant gymnosperms, with 627 species belonging to 70 genera, which are distributed worldwide (Farjon 2008). Several studies have shown that they are composed of seven families based on morphological-cladistic analysis (Hart 1987) and molecular phylogenetic analyses (Chaw et al. 1993; Price et al. 1994; Owens and Simpson 1999). These studies also show that Pinaceae is the first lineage of conifers to diverge. Other morphological analyses consider Phyllocladaceae as separate from Podocarpaceae resulting in eight families (Tomlinson et al. 1997; Farjon 1998, 2008). The conifers are monophyletic and starting from the basal branch, are composed of Pinaceae, Podocarpaceae sensu lato, Araucariaceae, Sciadopityaceae, Taxaceae, Cephalotaxaceae, and Cupressaceae (Stefanovic et al. 1998). The placements of Pinaceae and Podocarpaceae in the above and many other molecular phylogenetic analyses are not consistent with the fossil record of the conifers (Stewart 1983; Farjon 1998, 2008). Therefore, the number of conifer families and their position in an evolutionary classification remain to be settled.

The gnetophytes have been grouped within the gymnosperms based on their naked ovules. The gnetophytes have also been regarded as a sister group to the angiosperms due to many shared morphological characteristics including double fertilization (Friedman 1990a, 1990b; Carmichael and Friedman 1996). However, several studies indicate otherwise (Goremykin et al. 1996; Chaw et al. 1997; Hansen et al. 1999). Sequence analyses of mitochondrial and nuclear small subunit rRNA (Bowe et al. 2000) and examination of molecular markers such as the MADS-box gene subfamilies (Winter et al. 1999; Chaw et al. 2000) have provided further evidence that gnetophytes are monophyletic with a closer affinity to the conifers than angiosperms. Moreover, sequence information from RNA polymerases I, II, and III has grouped the gnetophytes within the conifers as the sister group of the Pinaceae (Hajibabaei et al. 2006). The fossil record also provides some evidence for a relationship between the Coniferales and Gnetales (Hernandez-Castillo et al. 2001).

In spite of the discrepancies in the placement of Gnetales, it is agreed that three genera compose this order: Ephedra, Gnetum and Welwitschia. Although different in appearance and life history, the three genera are grouped together based upon shared morphological characteristics including vessels in the secondary wood, compound cones in both male and female plants, double-integumented ovules, long micropylar tubes formed by the inner integuments, double fertilization, embryos by two cotyledons, opposite and net-veined leaves, and lack of resin canals. Most of these features are not found in any other gymnosperms. The monophyly of these three genera are also supported by various molecular data (Goremykin et al. 1996; Price 1996; Bowe et al. 2000; Chaw et al. 2000; Rydin et al. 2006).

**MORPHOLOGY AND POLARITY OF POLLEN GRAINS**

Pollen grains are produced by all seed plants and generally range in size from 10-100 microns. In gymnosperms, pollen grains represent the developing male gametophytes that are surrounded by a complex wall, the pollen wall, composed of an outer layer called the exine (which is subdivided into ectexine and endexine) and the inner layer is the intine (Faegri and Iverson 1989). The ectexine is relatively thick and composed primarily of sporopollenin (which is the most decay- and chemical resistant biopolymer available in nature), while the endexine is thin, with a nonhomogenous laminate appearance, and degradable. The exine protects the developing male gametophyte from impacts and abrasion, and allows for the expansion and reduction of the pollen grain size with changing humidity. The intine is composed of various proteins and polysaccharides, and plays a role in pollen germination and extension of the pollen tube (Derkson et al. 1999; Yatomi et al. 2002; Chichiricco and Pacini 2008).

The pollen grains of cycads (Fig. 1A), which probably represent the nearest living equivalent to the earliest gymnosperm pollen grain (Chaloner 1970), are smooth, spheroidal, and aperturate; the aperture occupies almost half of the pollen surface (Dehgan and Dehgan 1988; Tekleva et al. 2007). Recent studies place those with small volume, such as in Encephalartos where the volume is approximately 7 µm³ (Pacini et al. 1999), to those with large volumes, such as in Macrozamia and Microcycas with volumes of approximately 31 µm³ (Pacini et al. 1999). Polarity is manifested in the thickness of the outer wall of the pollen grain. The exine covers the entire surface of the pollen grain; however, it is thickest at the proximal area, moderate on the sides, and thin at the distal area. Proximal refers to the surface where the microspores join in the tetrad following meiosis and distal refers to the outer surfaces of the four microspores when in the tetrad and after they separate. The intine is a single, thin, continuous layer (although it is thicker on the sides in some species) composed of cellulose, callose, and pectins (Downie 1928; Pettitt 1982; Pacini et al. 1999; Yatomi et al. 2002). The intine is made up of a thick inner layer rich in cellulose and a thinner outer layers which may be saccate or non-saccate, with smooth, orbiculate, or highly sculptured walls (Fernando et al. 2005a). The most detailed ultrastructural description yet made of the formation of all layers of the conifer pollen wall, including for each layer the structure, terminology, and the sequence of deposition has been made for Tsuga canadensis by Kurmann (1990). Therefore, readers are referred to this and other articles by this author for details on pollen wall structure in other conifers (Kurmann 1994), as well as comparisons among extant gymnosperms (Kurmann 1994), the angiosperms (Kurmann and Zavadil 1994), and between extant and fossil gymnosperms (Kurmann and Zavada 1994). In most Pinaceae, pollen grains are saccate and strongly polarized by the presence of distal sacci (Fig. 1C) (Owens and Simpson 1986). The exine is thicker at the proximal end and thinner but continuous in the distal region. The cellulose-rich intine contains the prothallial cells at the proximal end. In other Pinaceae (e.g., Pseudotsuga and Larix), pollen grains are non-saccate, are very finely sculptured (Fig. 1D), and lack external polarity, but have internal polarity in the presence of prothallial cells embedded within the intine. In the Cupressaceae, pollen grains are spherical, smooth, non-saccate and the exine surface bears many tiny spherical orbicules (Fig. 1E) deposited from the tapetum. In Tsuga (Pinaceae), the pollen grains are also non-saccate but the exine forms spines (Fig. 1F). Their spherical pollen grains become irregularly indented when dry and are internally polarized (Owens and Simpson 1986). The cellulose-rich inner layer of the intine is uniform in thickness, while the outer layer, which is rich in pectins, is irregular in thickness (Chichiricco and Pacini 2008). In contrast, pollen grains of the Taxaceae lack internal polarity. Their exine is continuously thick as is the intine, which is made up of a thick inner layer rich in cellulose and a thinner outer layer containing pectin (Anderson and Owens 2000; Fernando et al. 2005a). Their pollen is non-saccate and also covered with orbicules. Saccate pollen grains are also found
in most Podocarpaceae (Salter et al. 2002).

The pollen grains of Ephedra and Welwitschia are ellipsoidal (El-Ghazaly et al. 1997; Rydin and Friis 2005), while it is generally subspherical in Gnetum (Kurmann 1991; Yao et al. 2004) (Figs. 1G-J). The pollen wall surface of Ephedra and Welwitschia are polyplicate, i.e., characterized by a series of longitudinal ridges, while it is spinulose in Gnetum (Yao et al. 2004). In Ephedra, the exine has alternating thick and thin areas, while the cellulose-containing intine is relatively thick and evenly surrounds the entire pollen grain (El-Ghazaly et al. 1997; Rydin and Friis 2005). The volumes of Ephedra pollen grains have been estimated at 18 μm³ (Pacini et al. 1999) and internal polarity is manifested by the placement of the prothallial cell at the proximal end (El-Ghazaly et al. 1997). The pollen grains are inaperturate in Ephedra (El-Ghazaly et al. 1997) and Gnetum (Yao et al. 2004), while monoaperturate in Welwitschia (Rydin and Friis 2005).

CELLULAR COMPOSITION OF POLLEN GRAINS

In seed plants, development of male gametophytes begins with haploid microspores, which are products of meiosis by microspore mother cells that occur within microsporangia. For general descriptions of the initiation and development of the microsporangium and microsporogenesis in various gymnosperms, readers are referred to Singh (1978) and Gifford and Foster (1989). In gymnosperms, pollen grains are dispersed at the one- to five-cell stage (Fernando et al. 2005a), except in podocarps where the prothallial cells may proliferate and so their pollen grains may be shed with as many as 40 cells or nuclei (Chamberlain 1935; Sterling 1963; Quinn 1964; Gifford and Foster 1989; Wilson and Owens 1999). Terminologies used to describe cellular compositions both within the pollen grain and tubes have been inconsistent among various accounts of male gametophyte development for gymnosperms over the last many decades. This has led to confusion and misinterpretations by authors in
Fig. 2 Male gametophyte development in cycads.

Fig. 3 Male gametophyte development in Ginkgo.

many articles over the years (for a complete review, see Singh 1978; Gifford and Foster 1989; Owens and Bruns 2000). In spite of the efforts by many authors (Chamberlain 1935; Sterling 1963; Singh 1978; Gifford and Foster 1989; Owens and Bruns 2000; Fernando et al. 2005a) to standardize the terminology, terms used are still not consistent among some authors and even with the same author when describing a different gymnosperm. We believe that terminologies can be standardized and applied to all gymnosperms. Consistent use of terms will facilitate comparison of the whole process of male gametophyte development, not only within the gymnosperms, but also between gymnosperms and angiosperms.

The rationale behind our choice of terminologies is based on the assumption by Friedman and Gifford (1988), that the evolution of the male gametophyte and its sexual organ (antheridium) involved a significant reduction and simplification. In gymnosperms, prothallial cells are among those that have been reduced in number or eliminated. In fact, these cells have been completely eliminated in the male gametophytes of angiosperms. The discussion that follows will show that there has been a further reduction in the number of cells that constitute the male gametophytes of gymnosperms, particularly in some conifers and gnetophytes.

In this review, we present our recommended terminologies for the cells and nuclei involved in the sequence of mitotic divisions during the development of the male gametophytes in gymnosperms (Figs. 2-10). The following descriptions incorporate the recommended terms, followed by the previously used terms in parentheses, and where possible the rationale behind the changes. What is being proposed here is not the final solution to the problems described, but if it stimulates discussion on this subject, then this has achieved its goal.

A. In cycads (Fig. 2), the microspore undergoes an asymmetric cell division to form a small prothallial cell and a large antheridial initial (meristematic initial). The antheridial initial also divides asymmetrically to form a large tube cell and a small antheridial (generative) cell. Pollen grains are released with the three cells axially aligned with the flattened prothallial cell proximally situated, the antheridial cell centrally placed, and the large tube cell distally positioned (Singh 1978; Gifford and Foster 1989; Norstog 1990; Ouyang et al. 2004).

We support the use of the term antheridial initial by Singh (1978) instead of “meristematic initial or cell” by Gifford and Foster (1989). The cell under discussion does not give rise to any prothallial cell and is, therefore, the very first cell of the sperm lineage (or antheridium) and referring to it as the “antheridial initial” is appropriate. Gifford and Foster (1989) used the term “meristematic initial or cell” for the cell under discussion, but they also used it to describe the cell giving rise to the antheridial initial and second prothallial cell, resulting in confusion. Furthermore, their term conflicts with the concept of a meristem, as used to describe the origin of roots, shoots, secondary vascular tissues, and secondary protective tissues. Therefore, to avoid any confusion, we do not recommend the use of the term “meristematic initial or cell.”

We also support the term “antheridial cell” by Singh (1978) instead of “generative cell” by Gifford and Foster (1989) to refer to one of the cells (in addition to the tube cell) produced by the division of the antheridial initial. We believe that the term “generative cell” should only be used to describe the cell that gives rise to the sperm.

B. In Ginkgo (Fig. 3), the microspore undergoes uneven cell division to produce a small and flattened first prothallial cell and a large central cell (meristematic initial). Unequal division of the central cell forms a second small prothallial cell and a large antheridial initial. The latter subsequently divides forming a tube cell and an antheridial (generative) cell, which is in contact with the second prothallial cell. The pollen grains are shed at the four-cell stage, composed of two prothallial cells, an antheridial cell, and a tube cell in an axial row (Singh 1978; Gifford and Foster 1989; Friedman 1987).

We support the use of the term “central cell” as used by Singh (1978) instead of “meristematic initial” according to Gifford and Foster (1989) because the former is descriptive of the position of the cell under discussion, whereas the latter is problematic as described earlier. Singh (1978) used the term “central cell” to replace “embryonal cell” (as used previously by Sterling 1963), which he considered unsuitable since it implies a relationship with the embryo, and we agree.
Four patterns of cell division are recognized in conifer pollen grains (Owens and Bruns 2000; Fernando et al. 2005a), with some families containing different numbers of cells at dehiscence (Figs. 4-7). The following sequence of conifer families is based in the complexity of cell divisions starting from those families with the greatest number of cell divisions prior to dehiscence and sperm formation. This sequence of conifer families will be used from this point on.

C. In Podocarpaceae and Araucariaceae (Fig. 4), the sequence of cell divisions is essentially similar to Ginkgo except that the first and second prothallial cells may undergo further divisions resulting in a variable number of prothallial cells or nuclei, which are appropriately referred to as primary and secondary prothallial cells, respectively. The term “primary prothallial cells” is used to differentiate these cells from those that they produce, i.e., the “secondary prothallial cells”.

In Araucaria, prothallial cells form a tissue-like tier of cells and subsequently their cell walls and membranes break down resulting in many free prothallial nuclei in the tube cell cytoplasm. In Podocarpus, six to eight prothallial cells are formed (Quinn 1964; Wilson and Owens 1999), while as many as 40 cells or nuclei are formed in Agathis (Chamberlain 1935; Sterling 1963; Gifford and Foster 1989). According to Owens et al. (1995), most prothallial cells in Agathis are bound by cell membranes and not cell walls, and appear almost as free nuclei. The unequal division of the antheridial initial then forms a large tube cell and a smaller antheridial cell. The antheridial cell divides to form a sterile (stalk) cell and a generative (body or spermatogenous) cell. The pollen grains are shed at the four- (Singh 1978; Gifford and Foster 1989) or five-cell stage (Pettiti 1985; Said 1989; Owens and Bruns 2000; Fernando et al. 2005a).

Singh (1978) referred to the stalk cell in Pinaceae as such because of its position between the second prothallial cell and the generative cell. However, its position relative to the generative cell is reversed in the cypress Athrotaxis (Brennan and Doyle 1956). Gifford and Foster (1989) believed that the term suggests an unproved homology with the stalk of the antheridium of bryophytes and some leptosporangiate ferns. On the other hand, since the “stalk cell” in some gymnosperms is nonfunctional and therefore, sterile, it was called a “sterile cell” by Sterling (1963) and Gifford and Foster (1989), which we also consider as more appropriate. In addition, similar sterile cells occur in other conifers where they have no consistent position and may be free within the pollen tube cell cytoplasm. An exception to the concept of a sterile cell occurs in the cycad Microcycas, where the cell under discussion divides repeatedly and eventually giving rise to many sperm (Downie 1928). The term “secondary prothallial cell” has also been suggested by Singh (1978) to describe the cell under discussion, but this term implies that it is derived from other prothallial cells, which is incorrect. The term “sterile cell” presents fewer problems and was preferred by Owens and Bruns (2000) and Fernando et al. (2005a), and therefore, we suggest continuing the use of this term.
In early gymnosperm literature as discussed by Singh (1978), the term “body cell” was used for the cell that formed two sperm. However, this term has no functional or positional meaning and we recommend that throughout the gymnosperms that the term “generative cell” be used for the cell that divides to form the two sperm, in consonance with angiosperms. Singh (1978) considered the terms “body cell”, “spermatogenous cell”, and “generative cell” as being morphologically equivalent. Rudall and Bateman (2007) also considered the terms “spermatogenous cell” and “generative cell” as equivalent. Although Gifford and Foster (1989) considered the term “spermatogenous cell” to be more explicit than generative cell in denoting the function of the cell under discussion, several authors (Southworth and Cresti 1997; Owens and Bruns 2000; Fernando et al. 2005a) have begun to use the term “generative cell” for the cell in gymnosperms that forms the two sperm. Since this is consistent with the terminology used for angiosperms, we recommend that this trend be continued.

E. In Taxaceae and Cephalotaxaceae (Fig. 6), the microspores do not divide prior to being shed. Therefore, at pollination, microspores are also regarded as pollen grains, which are shed at the one-cell stage (Anderson and Owens 2000; Wang et al. 2008).

F. In Cupressaceae (Fig. 7), the microspore divides asymmetrically to form a large tube cell and a smaller generative cell. Cell divisions involved in the formation of the central cell, antheridial initial, and antheridial cell appear to have been eliminated. Therefore, the pollen grains are shed at the two-cell stage (Singh 1978; Fernando et al. 2005a; Chichiricco and Pacini 2008).

G. In Ephedra (Fig. 8), the pattern of male gametophyte development is most similar to Ginkgo and Pinaceae. A major difference from these gymnosperms is that during the formation of the so-called second prothallial cell, no cell wall or membrane is laid down to separate it from the antheridial initial and therefore, only a prothallial nucleus is formed (Friedman 1990b). Another difference is that both the first formed prothallial cell and the prothallial nucleus begin to break down soon after their formation (Singh 1978; Gifford and Foster 1989; Friedman 1990b). Division of the antheridial initial forms a tube cell and an antheridial (generative) cell. The latter divides to form a sterile nucleus and a generative (spermatogenous) nucleus, which are not separated by a cell wall or membrane and thus, should be referred to as nuclei that share a common cytoplasm (Friedman 1990b). Therefore, at dehiscence, the pollen grains consist of two cells and three nuclei.

H. In Gnetum and Welwitchia (Fig. 9), the microspore divides unequally to produce a small prothallial cell and a large antheridial cell (antheridial initial). The latter divides
Fig. 10 Sequence of cell divisions, using proposed standard terminology, in male gametophyte development in extant gymnosperms. The previous terminology is shown in parentheses. The dashed lines indicate the stage of pollen grain dispersal. AI, antheridial initial; BC, body cell; CC, central cell; EC, embryonal cell; GC, generative cell; MI, meristematic initial; PC1, first prothallial cell; PC2, second prothallial cell; PC2, secondary prothallial cell; PN, prothallial nucleus; SC, sperm cell; SN, sperm nucleus; STA, stalk cell; SPC, spermatogenous cell; STE, sterile cell; STN, sterile nucleus; TC, tube cell.
to form a tube cell and a generative cell. As compared to Ephedra, only the cell divisions involved in the formation of the central cell and antheridial initial appear to have been eliminated in Gnetum and Welwitschia. Therefore, the pollen grains in these genera are shed at the three-cell stage (Singh 1978; Carmichael and Friedman 1995).

Based on the above, two patterns of cell division are recognized in the pollen grains of Gnetophytes (Singh 1978; Gifford and Foster 1989), with the three genera containing different numbers of cells and nuclei at pollination.

**Fig. 10** is provided to facilitate comparison of the sequences of mitotic divisions during the development of the male gametophytes in all four living phyla of gymnosperms.

### POLLEN GERMINATION AND SPERM FORMATION

A major difference in development of male gametophytes in gymnosperms and angiosperms is that in the former, no pollen grains are shed with sperm already present. Therefore, as far as we are aware, sperm formation in gymnosperms occurs exclusively after pollination. To emphasize this difference, the description of the development of male gametophytes after pollination is separated from the description of the development before pollination, and therefore provided under a different heading. In this case, some overlaps will be noticed but are intentional.

After pollination, pollen grains germinate outside or inside the ovule through various mechanisms. For detailed description of this subject, readers are referred to Singh (1978), Tomlinson (1994), Owens et al. (1998) and Labandeira et al. (2007). Post-pollination development of the male gametophyte is manifested by the formation and elongation of the pollen tube. At this stage, prothallial and sterile cells in some genera may migrate into the elongating pollen tube; however, the information on this subject is incomplete to draw any meaningful generalizations. Most authors did not provide information on the activity of prothallial cells probably because these cells do not have any known function in gymnosperms. The discussion on these cells will be limited to a few well-established examples. There is confusion as to the nature of the sperm in gymnosperms. To facilitate our understanding of this subject, examples will be described to show which gymnosperms have sperm cells and which ones have sperm nuclei. Therefore, this section will focus on the activity of the generative cell, time of sperm formation and nature of the sperm (Fig. 11A-I).

#### A. In cycads (Fig. 2), as pollen grains are brought into the pollen chamber at the tip of the nucellus, they absorb water and nutrients from the pollen chamber. This hydration stimulates germination within the chamber, which may not occur until months after pollination. After the pollen tube forms from the three-cell pollen grain, the antheridial cell divides to produce a sterile cell and a (spermatogenous) generative cell, after which the prothallial cell enlarges causing it to press against the sterile cell (Swamy 1948; Ouyang et al. 2004). Division of the generative cell results in two sperm cells, each enclosed by a flagellum and approximately 40,000 flagella each formed by a cluster of blepharoplasts; the sperm cells only have cell membranes and their nuclei comprise the bulk (Norstog et al. 2004; Rudall and Bateman 2007).

It is interesting to note that in Microcycas, the so-called sterile cell may divide to form rows of generative cells that develop into at least sixteen flagellate sperm cells (Downie 1978). Normal division of two sperm cells in Microcycas reflects a plesiomorphic state in male gametophyte evolution (Norstog 1990; Norstog and Nicholls 1997). However, morphological and molecular evidences have shown that Cycas is the basal genus (Stevenson 1992; de Laubenfels 1999; Hill et al. 2003) implying that polyploidy is derived. In fact, polyploidy has been regarded as atavistic reversal (Norstog et al. 2004; Rudall and Bateman 2007).

#### B. In Ginkgo (Fig. 3), about a week after pollen grains are drawn into the ovule, germination occurs (Friedman 1987). When the four-cell pollen grain germinates, the first prothallial cell aborts while the second prothallial cell remains intact. The antheridial cell divides to produce a sterile cell and a generative (spermatogenous) cell. The latter is situated between the second prothallial cell and the sterile cell. The generative cell is believed to share a common cell wall with the second prothallial cell (Lee 1955). During sperm formation, that occurs during cell-division and two blepharoplasts form, where one is included in each of the two sperm cells (Fig. 11B). In each of the sperm cells, the blepharoplast gives rise to the flagellar apparatus that contains approximately 1000 flagella (Gifford and Lin 1975). The two sperm cells are contained within the generative cell cytoplasm, but are eventually released (Singh 1978; Gifford and Larson 1980; Friedman 1987).

There are at least two differences between the generative cells of Ginkgo and cycads. In Ginkgo, the cytoplasm contains a lens-shaped nucleus flanked by a pair of globular or osmiophilic bodies (Gifford and Lin 1975), while in cycads, nuclei are spherical with no such bodies observed (Norstog and Nicholls 1997).

In conifers (except Taxaceae and Cephalotaxaceae) and gnetophytes, as compared to the cycads and Ginkgo, the pollen grain germination during post-pollination development of the male gametophytes is the formation of the sperm from the generative cell (Figs. 4-9). The pollen grains in conifers germinate upon hydration followed by the shedding of the exine or penetration of the pollen tube through a thin area in the exine (Owens et al. 1998). Pollen germination occurs one (Fernando et al. 2005b) to several weeks (Owens et al. 1994; Takaso and Owens 1997) after pollination, prothallial cells usually do not migrate into the elongating pollen tube.

#### C. In Podocarpaceae and Araucariaceae (Fig. 4), during germination of the typical five-cell pollen grain, the nucleus of the generative cell divides to form two sperm nuclei (Fig. 11C-D), which remain within the generative cell cytoplasm. Two differences occur within the Araucariaceae but not in the Podocarpaceae: 1) the generative cell that enters one of the branches leading to an archegonium divides to form two equal-size sperm nuclei that are contained within the generative cell cytoplasm; and 2) during sperm formation, the two nuclei that are formed engulf the generative cell cytoplasm and its organelles forming two large complex sperm nuclei (Owens et al. 1995). The latter has not been reported in Podocarpaceae and that the sperm nuclei that are formed are unequal in size (Wilson and Owens 1999).

#### D. In Pinaceae (Fig. 5), after germination of the five-cell pollen grain, the nucleus of the generative cell divides to form two sperm nuclei (Figs. 11E-F). The prothallial cell remains at the tip of the pollen tube until the pollen tube enters the egg cell. In most species studied, no cell wall or membrane is formed between the two sperm nuclei (Fig. 11E) and, therefore, they share the organelles of the generative cell (Singh 1978; Owens and Bruns 2000). In Picea (Fig. 11F), only a partial cell wall is formed between the two sperm nuclei (Dawkins and Owens 1993; Runions and Owens 1999). In Taxaceae and Cephalotaxaceae (Fig. 6), the one-cell pollen grain upon entering the pollination drop germinates and then divides to form a large tube cell and a smaller antheridial cell. The latter then divides to form a sterile cell and a generative cell. These cells are both contained within the elongating pollen tube (Anderson and Owens 2000; Wang et al. 2008). In these families, the prothallial cells, central cell and antheridial initial appear to have been eliminated. During mature pollen tube development, the generative nucleus divides to form two sperm nuclei (Fig. 11G) that remain closely associated within the cytoplasm of the generative and sterile cells (Pennell and Bell 1986; Anderson and Owens 1999, 2000; Wang et al. 2008). In some cases, this association occurs even after the disintegration of the cell walls and membranes of the generative and sterile cells (Anderson and Owens 2000; Wang et al. 2008). Therefore, the sperm in these families are also nuclei that are surrounded typically by the generative cell cyto-
plasm.

F. In Cupressaceae (Fig. 7), after the two-cell pollen grain has germinated, the generative cell divides to form two nuclei and each becomes surrounded by cell walls and membranes forming two large equal-size sperm cells (Fig. 11H) that contain abundant organelles (Singh 1978; Fernando et al. 2005a). Therefore, unlike the sperm in other conifer families, which are made up of nuclei, sperm in Cupressaceae are made up of cells.

The development of the male gametophyte in Cupressaceae parallels that of angiosperms, particularly the eudicots. Considering that this family is one of the more derived groups in conifers (Chaw et al. 1997; Stefanovic et al. 1998) and not closely related to any of the angiosperms, such similarity in the development of the male gametophytes suggests a case of convergent evolution.

G. In Ephedra (Fig. 8), the exine is completely shed before pollen germination (El-Ghazaly et al. 1997). Germination of the five-cell pollen grain is followed by the division of the generative cell nucleus to yield two sperm nuclei that are contained within the generative cell. The generative cell containing the two sperm nuclei becomes tightly pressed to the sterile cell (Friedman 1990b).
H. In *Gnetum* and *Welwitschia* (Fig. 9), the prothallial cell degenerates before or after germination of the three-cell pollen grain. In the pollen tube, division of the generative cell nucleus produces two sperm nuclei (Fig. 11), which also remain within the generative cell cytoplasm. The generative cell containing two sperm nuclei becomes situated adjacent to the tube nucleus (Carmichael and Friedman 1991, 1996).

Based on the complete development of the male gametophytes, *Ephedra* exhibits a pattern that is distinct from those of *Gnetum* and *Welwitschia*. This trait adds further support to the position of *Ephedra* as a sister group to the *Gnetum-Welwitschia* clade (Doyle and Donoghue 1992; Goremynkin et al. 1996; Bowe et al. 2000; Chaw et al. 2000; Rydin et al. 2006).

**GROWTH OF POLLEN TUBES IN THE OVULE**

In most gymnosperms, pollen grains are taken into the ovule, usually by a pollination drop, and sink or are drawn to the surface of the nucellus where they germinate. The pollen tube is either zooidogamous, as in cycads and *Ginkgo*, acting as a haustorial organ and not directly responsible for sperm delivery to the egg cell, or siphonogamous, as in conifers and gnetophytes, delivering the sperm directly into the egg (Poort et al. 1996; Rudall and Bateman 2007).

The haustorial and branching nature of the pollen tubes in the cycads and *Ginkgo* may have been the result of the shift in habit of the male gametophyte from its prior free-living existence (as seen in bryophytes, lycophytes and pteridophytes) to one within the tissues of the sporophyte (Poort et al. 1996; Rudall and Bateman 2007). During early evolution of seed plants, it has been speculated that the “invasion” of the pollen tube into the nucellus may have elicited a host-pathogen response in the sporophyte (Choi and Friedman 1991). As a result, the pollen tube may have relied on branching and intracellular growth to gain nutrients. In the more derived groups, conifers and gnetophytes, the sporophyte may eventually have come to facilitate and assist the growth and development of the pollen tube thus, branching was reduced or lost (Choi and Friedman 1991).

A. In cycads, pollen germination involves exine rupture followed by the initiation of the pollen tube from the distal end of the pollen grain, which occurs by means of a protrusion of the intine through the germinal furrow of the monosulcate pollen grain (Pettitt 1982). The tube nucleus migrates into the elongating pollen tube, which begins to grow between degenerated nucellar cells lining the pollen chamber. As the pollen tube penetrates the nucellus, which is comprised of sporogenous cells, it accumulates its nutrient supply from the nucellar cells and the pollen tube becomes surrounded by a single layer of crushed nucellar cells. The abundance of nutrients in the nucellus may cause a response in the pollen tube, influencing the direction of its growth away from the archegonia. Upon reaching the subepidermal layer of the nucellus, the pollen tube becomes quite broad and at some point begins to produce horizontal outgrowths that penetrate adjacent nucellar cells, ultimately causing neosis of these cells. As the pollen tube elongates, it accumulates well-developed starch grains, while starch grains are nearly absent in the adjacent nucellar cells but abundant in the nucellar cells five or six cell layers away from the pollen tube. This suggests that the pollen tube absorbs substances from the surrounding sporophytic tissue to sustain its growth. This also suggests that enzymes secreted from the pollen tube outgrowths facilitate its growth and passage through the nucellus by digesting cell walls and protoplasts. Although it has not been demonstrated *in vivo*, Choi and Friedman (1991) detected polygalacturonases, cellulases, and other hydrolytic enzymes in the cyad pollen tube. Overall, the significant destruction of nucellar cells may compensate for the low surface area to volume ratio of the pollen tube, leading to greater access to nutrients (Choi and Friedman 1991). During pollen tube growth into the nucellus, the pollen chamber enlarges to form a cavity above the archegonial canal. The pollen tube at the proximal end of the pollen grain, where the prothallial and antheridial (generative) cells are located, enters this cavity and becomes swollen pushing the cells towards the pollen chamber. Here, the antheridial cell divides to ultimately produce the two multi-flagellate sperm cells. The swollen area of the pollen tube eventually ruptures and the sperm cells exit through a small orifice and proceed to swim to the egg (Swamy 1948). The growth of the pollen cell, causing the nucellus to typically be intercellular. However, the pollen tube also undergoes intracellular growth by production of localized outgrowths unique to this group.

B. In *Ginkgo*, development of pollen tubes occurs in three phases (Friedman 1987; Friedman and Gifford 1997): 1) initial diffuse and roughly isodiometric growth that starts at the distal end of the pollen grain; 2) tip growth during which the pollen tube elongates, branched or lost (Choi and Friedman 1991). As a result, the pollen tube may have remained within the tissues of the sporophyte (Poort et al. 2005a). In contrast, in some Pinaceae (e.g., *Pinus*, *Picea*, *Cedrus*, and some *Tsuga* species) and some Podocarpaceae, in which the ovules are inverted, pendant pollination drops form and extend out of the microphyte. The saccate pollen grains float up through the microphyte and up through the micropyral canal to the tip of the nucellus (Runions et al. 1995; Owens et al. 1998). There, they germinate with their pollen tubes growing through the nucellus and into an archegonium (Owens et al. 1998). In *Abies*, the pollen grain germinates in the micropyral canal.
and its pollen tube grows towards the nucellus as the nucel-
lus grows towards it, with the two structures meeting about
midway along the micropylar canal (Owens and Molder
1977; Chandler and Owens 2004). In Pseudotsuga, the pol-
len grain sheds its exine near the micropyle and elongates
along the length of the micropylar canal. A thin pollen tube
forms after the elongated pollen grain contacts or nearly
contacts the nucellus, then the pollen tube grows through
the nucellus as an archegonium (Owens et al. 1995).
Unlike the above taxa, the pollen grains in some Tsuga spe-
cies in the Pinaceae (Colangeli and Owens 1989) and all
species of Araucariaceae (Owens et al. 1995) germinate on
the surface of a bract, scale or integument and the pollen
tubes grow into the micropyyle, nucellus and eventually into
an archegonium. In Agathis (Araucariaceae), pollen grains
land and germinate on the nucellar tip that grows out through
the micropyyle. Once within the nucellus, the pollen tube in
the Araucariaceae forms many long branches; one of these
branches contains the generative cell and grows into one of
the several separate archegonia (Owens et al. 1995).

Slightly branched pollen tubes occur sporadically in
various conifers. In Pinus contorta, many short branches
(about 10 nucellar cells long) form after pollination and
during the first year of cone development (de Win et al.
1995; Owens et al. 2005). The pollen tube appears to be
haustorial but the tube nucleus usually enters one of the
longer branches before the cone becomes dormant. Follow-
ing winter dormancy, the branch containing the tube nu-
cleus elongates more rapidly than the other branches and
within a few weeks reaches an archegonium (Owens et al.
2005). The pollen tube branches in pines are less extensive
as compared to those in cycads and Ginkgo and some mem-
bers of the Araucariaceae (Owens et al. 1995). This indi-
cates that any haustorial role in conifer pollen tubes is at
best secondary (Rudall and Bateman 2007).

Pollen tubes may develop from both the proximal and
distal ends of pine pollen grains, but only the pollen tube
at the distal end develop further, just like in other conifers
(Fernando et al. 2005a). Conifer pollen tubes grow through
the nucellus prior to egg maturation (Takaso et al. 1996).
Fertilization occurs as little as a few weeks after pollination
in most Cupressaceae and Pinaceae (Bruns and Owens
2000), but about a year after pollination in Pinus and some
Araucariaceae (Owens et al. 1995). The growth of the
pollen tube appears to be intercellular within the nucellus;
however, it is accompanied by modest degeneration of
adjacent nucellar cells. These cells likely degenerate due to
cell wall-acting enzymes secreted by the pollen tube that
enable the apex of the tube to push cells aside as it per-
meates the nucellus (Owens 1993). The collapse of the nu-
cellar cells facilitates the passage of the growing pollen
tube tip and their cellular contents may be utilized by the
developing pollen tube (Hiratsuka et al. 2002). In con-
fiers, proteins from pollen grain walls and pollen tubes are
believed to be involved in cellular degeneration (Pettitt
1985) and stress/defense responses among others (Fernando
2005). In Pinus contorta, each of the many short pollen
tube branches contains several nuclei which are believed
to trigger their collapse. The short elongating pollen
tube branches occupy the spaces left by the collapsed nu-
cellar cells (Owens et al. 2005).

D. In Ephedra and Gnetum, after the pollen grains are
brought into the ovule, they shed their exine upon germina-
tion (El-Ghazaly et al. 1997). In Welwitschia, the sulcus
slits open and the pollen tube extends out of the exine,
which remains as a cap-like structure after covering
the developing pollen tube (Rydin and Friis 2005).
There are fewer studies on the development of the male
gametophyte in gnetophytes compared to other gymno-
spers, yet it appears that their pollen tubes are simple and
unbranched. The pollen tubes arise only from the distal end
of the pollen grain. As the pollen tube elongates, it passes
through the intercellular spaces between the nucellar cells
without disrupting their cellular integrity. In Ephedra, when
the pollen tube arrives at an archegonium, a vaculated
region (fertilization chamber) forms at the apex into which
the pollen tube releases the tube nucleus, sterile cell, and
the two sperm nuclei. The two sperm nuclei enter the egg
cytoplasm, one fuses with the egg nucleus and the second
with a sister nucleus, the ventral canal nucleus, which re-
sults in a double fertilization event unique among the gymn-
ospers (Friedman 1990a, 1990b). In Gnetum and Welwit-
schia, the nuclei within the female gametophytes do not
undergo further differentiation and therefore, archegonia are
not produced (Singh et al. 1978). In all gnetophytes, multiple
pollen tubes may develop and reach the archegonia or
female gametophytes. In Ephedra, only the first pollen tube
to reach an archegonium will release the sperm nuclei; the
supernumerary pollen tubes do not grow further and their
contents become impounded in the fertilization chamber.
However, in Gnetum, multiple fertile nuclei are available in
the female gametophyte for fertilization since no egg cell
differentiates and, therefore, many or all of the pollen tubes
can successfully develop and deliver sperm nuclei for fer-
tilization (Carmichael and Friedman 1996). In Welwitschia,
multiple fertile nuclei are also available for fertilization, but
these are contained in prothallial tubes that elongate from
the micropylar end of the female gametophyte to about half-
way through the nucellus (Singh 1978).

The time interval between pollination and fertilization
(fertilization internal) in gymnosperms ranges from 10 h
to more than 12 mo. In general, pollen hydration and germina-
tion takes two or more days, and the period of active growth
of the pollen tube five days or more. Except for Gnetum and
Ephedra, which have a fertilization interval of 6-8 days
and 10-36 h, respectively, the fertilization interval in gymno-
spers is much longer as compared to angiosperms (15 min
to >12 mo). In Ephedra, this short interval is accounted for
by the shortening of the pollen tube pathway and a decrease
in the pollen germination time of about 1-2 h (Williams
2008). However, like other gymnosperms, the rate of pollen
tube growth, ~14 μm/h, in vivo, is slow compared to that
observed in angiosperms, which ranges from ~80-600 μm/h.
The maximum rates of pollen tube growth in other gymno-
spers have been measured in vivo: Zamia, ~1 μm/h; Ginkgo,
2 μm/h; Agathis, 6 μm/h; and Gnetum, 5 μm/h; and
in vitro: Pinus, ~1 μm/h (Williams 2008).

COMPOSITION OF INTINE AND POLLEN TUBE
WALL

A few reports have focused on the chemical composition
of the intine and pollen tube wall in gymnosperms, and the
most comprehensive of these is that of Yatomi et al. (2002).
These authors examined 14 species belonging to eight
genera and six families representing cycadophytes, ginkgo-
phytes, and coniferophytes to localize arabinogalactan pro-
teins (AGPs), β-glucans (cellulose and callose) and pectins
(acidic and esterified) in both the intines and pollen tube
walls. Their results suggest that intines and pollen tube
walls of all the gymnosperms examined have AGPs and cel-
ulose microfibrils are oriented parallel and transverse to
the elongation axis of the pollen tube. This orientation is
less dense as

The occurrence of callose appears to be variable in
conifers. Yatomi et al. (2002) showed that callose MAb
strongly labeled the outer layer of the intines of all the six
pine species, Podocarpus macrophyllus, Cryptomeria japo-
nica and Chamaecyparis obtusa; strong labeling in the pol-
len tube wall was only observed in Cryptomeria japonica,
but slight labeling of the pollen tube wall was also observed
in most of the conifers examined. Using antilne blue to
detect callose, only the pollen tube walls of Podocarpus

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Callose plugs have not been reported in any gymnosperm (Yatomi et al. 2002). Also based on aniline blue staining, Derksen et al. (1999) showed that in P. sylvestris, callose is present in the pollen tube tip and younger parts of the pollen tube, but not in the older and perhaps, none elongating part of the pollen tube. In germinating Cupressus arizonica pollen, aniline blue staining was observed in the intine, pollen tube wall, and wall separating the generative and tube cells (Chichiricco et al. 2009).

Acidic and esterified pectins have been localized in the pollen tube walls and tips, respectively, in P. sylvestris (Derksen et al. 1999) and Picea wilsonii (Sheng et al. 2006). From the results of Yatomi et al. (2002), only P. nagi showed strong labeling with esterified pectin in both its intine and pollen tube wall, while in most of the other conifers, esterified pectin is more prevalent in the intines than in the pollen tube walls. Unfortunately, Yatomi et al. (2002) did not differentiate the localization of pectins between the pollen tube tip and the rest of the pollen tube wall.

The intines and pollen tube walls of Cycas revoluta and Ginkgo biloba differ from each other and the conifers in callose, pectin, and β-glucan compositions. In C. revoluta, callose has been detected in its intine, but not in its pollen tube wall, while callose staining is very weak in both the intine and pollen tube wall of G. biloba (Yatomi et al. 2002). Esterified and acidic pectins are found in both the intine and pollen tube wall of C. revoluta, whereas only esterified pectin is found in both the intine and pollen tube wall of G. biloba. No reaction was observed in both the intine and pollen tube wall of C. revoluta using a mixed glucan MAb (Yatomi et al. 2002). On the other hand, the strong reaction of a mixed glucan MAb observed in the intine and pollen tube wall of G. biloba suggests the presence of a β-glucan that is neither cellulose nor callose.

There is no information available on cyto- and immunochronical analyses of the intines and pollen tube walls in gnetophytes. Nevertheless, arabinoalagan proteins and cellulose are present in the pollen tube walls of the gymnosperms examined, pectin is prevalent in C. revoluta, and a β-(1,3)(1,4)-glucan is abundant in G. biloba. Callose has not been detected in the pollen tube walls of C. revoluta and all species of Pinus that have been examined, while detected in G. biloba and some conifers but at varying intensities. Callose plugs have not been reported in any gymnosperm and therefore, the entire pollen tube, even if it is extensively branched, remains as one continuous cell from germination through fertilization.

Maintenance of the pollen tube wall involves the formation, transport, and breakdown of many proteins. In conifers, by understanding the transport pathways, the importance and role of some proteins has been demonstrated (Wang et al. 2005). Pollen tube elongation, in particular, requires the assembly of structural proteins into the walls, which are synthesized in the ER-Golgi system and transported into the apoplastic space via secretory vesicles. The cell wall components become modified if the production and transport of these vesicles is blocked. The introduction of brefeldin A into germinating pine pollen by spheroplast injection is to inhibit the formation of the transport vesicles of the Golgi apparatus (Herr et al. 1993). The pollen tube grows in the absence of brefeldin A in germinating pine pollen, suggesting that a consistent balance of exocytosis and endocytosis is necessary for proper growth. Disruption of this balance causes a decrease in protein synthesis leading to the arrest of pollen tube growth.

MADS-BOX GENES AND REPRODUCTIVE DEVELOPMENT

Recent studies have given insights into the involvement of MADS-box genes in plant reproductive development. MADS-box is a conserved sequence motif found in a family of transcription factors from fungi, animals and plants (Alvarez-Buylla et al. 2000). While the reproductive structures of gymnosperms differ greatly from those of angiosperms, some gymnosperm genes have been found to possess MADS-box genes which are orthologous to angiosperm B- and C-class floral organ identity genes including Pinus radiata – MADS, Pinus resinosa – MADS, Picea abies – DAL1, 2; Picea mariana – SAG1; Ginkgo biloba – GBM5; Cycas edentata – CyAG; and Gnetum gnemon – GGM1, 2, 3, 9, 13 (Rutledge et al. 1998; Winter et al. 1999; Svenson and Engrström 2002; Nam et al. 2003; Jager et al. 2003; Zhang et al. 2004). As in angiosperms, these genes are believed to function in determining reproductive versus vegetative structures and male versus female reproductive units. Both orthologs found in Picea are expressed solely in the male and female reproductive tissues (Tandre et al. 1995; Tandre et al. 1998). Likewise in Gnetum, GGM5 is not expressed in vegetative tissues, but present only in the ovule and antherophore (pollen-bearing structure) (Winter et al. 1999; Becker et al. 2003). In Cycas, CyAG is present in the ovule and megasporophyll in the female, and in the male tissues, in the central axis of the cone, microsporophyll, and microsporangium (Zhang et al. 2004). Unlike the gymnosperm AGAMOUS, the GBM5 gene of Ginkgo is expressed not only in reproductive tissues, but also strongly in young leaves (Jager et al. 2003). In contrast to angiosperms, which are characterized by the presence of at least two genes belonging to the AGAMOUS (AG) family (C-class genes), only a single AG ortholog has been found in gymnosperms thus far (Jager et al. 2003). Further analysis of MADS-box genes including a larger number of gymnosperm representatives, may help in understanding the contribution of MADS-box genes in gymnosperm reproductive development.

Similar to angiosperms, the coupling of B gene and C gene expression is believed to control the formation of the male organs in gymnosperms (Theissen et al. 2000). Therefore, several studies have screened for genes containing MADS-box elements expressed in gymnosperm cones (strobili). Becker et al. (2003) have predicted that there is not one, but two B genes, GGM2 and GGM15, in Gnetum that are strongly expressed specifically in male structures. Futamura et al. (2008) have identified twelve MADS-box genes from the cDNA library of Cryptomeria japonica male cones, one is a type I gene and the other eleven are MIKC-type genes. These MIKC-type genes make up five subfamilies including DEF/GLO/GG13- (B-class and Bistre MADS-genes), TM5-, AG-, AGL16-, and TMS-like genes. The function of the type I gene remains to be determined in P. abies, some of which are active in all tissues of the developing cone, including the meristem, while DAL13 is expressed specifically in male cones. Likewise, MADS-box genes have been identified in the cones of Picea abies, many of which are related to the class B2 and C genes in angiosperms. Tandre et al. (1998) showed that DAL2, expressed in both male and female cones, is similar in structure and function to the class C MADS-box genes in angiosperms, and therefore is suspected to act as a determinant of reproductive organ identity. Similarly, the MADS-box gene DAL3, related to the angiosperm B-class genes, is also active in all tissues of the developing cone, including the meristem, while DAL1 is only active in the peripheral zone of the pollen cone bud (Sunström and Engrström 2002). In addition, there is evidence to suggest that the MADS-box gene of the gymnosperm MADS-box gene, a putative ortholog of the Arabidopsis genes AGL6 and AGL13, may have a role in regulating the juvenile-to-adult phase transition in P. abies. Active in the shoots of juvenile trees at 3-5 years of age, DAL1 expression increases with age, and is maintained at high levels in male and female cones. There are some MADS genes from gymnosperms that cannot be grouped with any MADS gene superfamilies from angiosperms, such as GGM7 in Gnetum (Becker et al.
2000). Likewise, the MADS-box gene DAL10 in P. abies shows expression specificity in developing seeds and pollen cones with no orthology to known angiosperm MADS clades (Carlesbecker et al. 2003). Therefore, it is likely that some MADS-box genes may be ancestral and lost during the evolution of angiosperms, whereas other may have been diverged after the separation of the angiosperms and gymnosperms.

While distinct MADS-box genes have been reported to show high expression levels in pollen grains, including AGL18 from Arabidopsis (Alvarez-Buylla et al. 2000), altogether the MADS family of genes is underrepresented in the pollen transcriptome. On the contrary, nonclassical lineages, including type I and MIKC*-type, have been found to be overrepresented in pollen grains. It is suggested that MIKC*-type genes play an essential role in late pollen development and pollen tube growth, as disruption of such genes is reported to have a negative effect on pollen maturation, competitive ability, and germination (Kofuji et al. 2003; Verelst et al. 2007a, 2007b; Adamczyk and Fernandez 2009). As these results have come mainly from angiosperms, there is a need to confirm the role of MIKC*-type genes in gymnosperm male gametophyte development. Characterization of these genes in gymnosperms may give further insight into their involvement in the evolution of male gametophytes in seed plants, as a whole.

**DIRECTION OF GYMNOSPERM MALE GAMETOPHYTE RESEARCH**

Studies on pollen grains and tubes in gymnosperms have been mainly confined to morphological, histological, and cytological analyses, particularly for cycads, Ginkgo, gnetophytes, and most conifers. There is no information available on the male gametophyte development in Sciadopityaceae and Phyllocladaceae. In spite of several decades of work on gymnosperm male gametophyte development and evolution, there is still a need to establish the morphological relationships between various cells comprising the male gametophytes within seed plants and with those of non-seed plants. A possible approach is to examine the expression of molecular markers in gymnosperms and non-seed plants that have been established in flowering plants which are specific for vegetative cells (including pollen tube) and reproductive cells such as generative and sperm cells (see Twell 1992; Mori et al. 2005; von Beser et al. 2006).

Advances in the study of gymnosperm pollen grains and tubes have come primarily from conifers, although they are very limited as compared to the publications on pollen grains and angiosperms. In gymnosperms, recent reports are all from conifers, and these deal with differential display protein analysis (Chen et al. 2006), regulation of Ca²⁺ uptake (Kong et al. 2006), cytoskeleton dynamics (Chen et al. 2006; Sheng et al. 2006; Chen et al. 2007; Zhang et al. 2007), pollen tube wall composition (Sheng et al. 2006; Chichirico and Paccini 2008; Chichirico et al. 2009), activities of sterile and pollen tube nuclei (Wang et al. 2008), and microarray and proteomic analyses (Fernando 2008). There are no studies involving both molecular biology and genetics in gymnosperm male gametophyte development, not surprising considering the long-genera- tion time of these seed plants.

In addition to the large-scale expressed sequence tags available from various conifers including Pimus taeda and Picea glauca (http://foresttree.org/Idb; Pavy et al. 2007) and Cryptomeria japonica (Putamura et al. 2008), there are also EST sequencing projects on other phyla of gymnosperms such as Ginkgo biloba (Brenner et al. 2003), Cycas rumphii (Brenner et al. 2005) and Gnetum gnemon (Brenner et al., unpublished results). However, these projects are not directly addressing pollen grain and tube development or evolution. So far, information on the genes and proteins expressed in gymnosperm pollen grains and tubes are from Pinus strobus (Fernando 2005), Picea meyeri (Chen et al. 2006) and Pinus taeda (Fernando 2008). We anticipate that as molecular data accumulate from various representatives of the four living orders of gymnosperms, evolutionary patterns regarding the development of pollen grains and tubes may emerge from comparative sequence analysis.

A comprehensive analysis of pollen grains and tubes from seed plants will facilitate our understanding of the differences between the penultimate and ultimate phyla of land plant evolution, i.e., the gymnosperms and angiosperms. To do so will require a large-scale identification of genes and proteins from the male gametophytes coupled with characterization of their expression patterns and functions at the molecular level. If initiated, such study will provide a remarkable advancement in our understanding of sexual reproduction in seed plants.

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