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Evolutionary development of the plant spore and pollen wall

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Abstract

Background and aims Many key innovations were required to enable plants to colonize terrestrial habitats successfully. One of these was the acquisition of a durable spore/pollen wall capable of withstanding the harsh desiccating and UV-B-rich environment encountered on land. The spores of 'lower' spore-bearing plants and the pollen of 'higher' seed plants are homologous. In recent years, researchers have begun to investigate the molecular genetics of pollen wall development in angiosperms (including the model organism *Arabidopsis thaliana*). However, research into the molecular genetics of spore wall development in more basal plants has thus far been extremely limited. This review summarizes the literature on spore/pollen wall development, including the molecular genetics associated with pollen wall development in angiosperms, in a preliminary attempt to identify possible candidate genes involved in spore wall development in more basal plants.

Presence in moss of genes involved in pollen wall development Bioinformatic studies have suggested that genes implicated in pollen wall development in angiosperms are also present in moss and lycopsids, and may therefore be involved in spore wall development in basal plants. This suggests that the molecular genetics of spore/pollen development are highly conserved, despite the large morphological and functional differences between spores and pollen.

Future work The use of high-throughput sequencing strategies and/or microarray experiments at an appropriate stage of 'lower' land plant sporogenesis will allow the identification of candidate genes likely to be involved in the development of the spore wall by way of comparison with those genes known to be involved in pollen wall development. Additionally, by conducting gene knock-out and gene swap experiments between 'lower' land plant species, such as the moss model species *Physcomitrella patens*, and the angiosperm model species arabidopsis it will be possible to test the role of these candidate genes.

Introduction

The colonization of land by plants in the Palaeozoic was a highly significant event in Earth's history, both from an evolutionary point of view and because it fundamentally changed the ecology and environment of the planet (Beerling 2007). Land plants evolved to form crucial components of all modern terrestrial ecosystems through evolutionary adaptations involving changes in anatomy, physiology and life cycle (Waters 2003; Menand *et al.* 2007; Cronk 2009). Key adaptations include rooting structures, conducting tissues, cuticle, stomata, and sex organs such as gametangia and spores/pollen.

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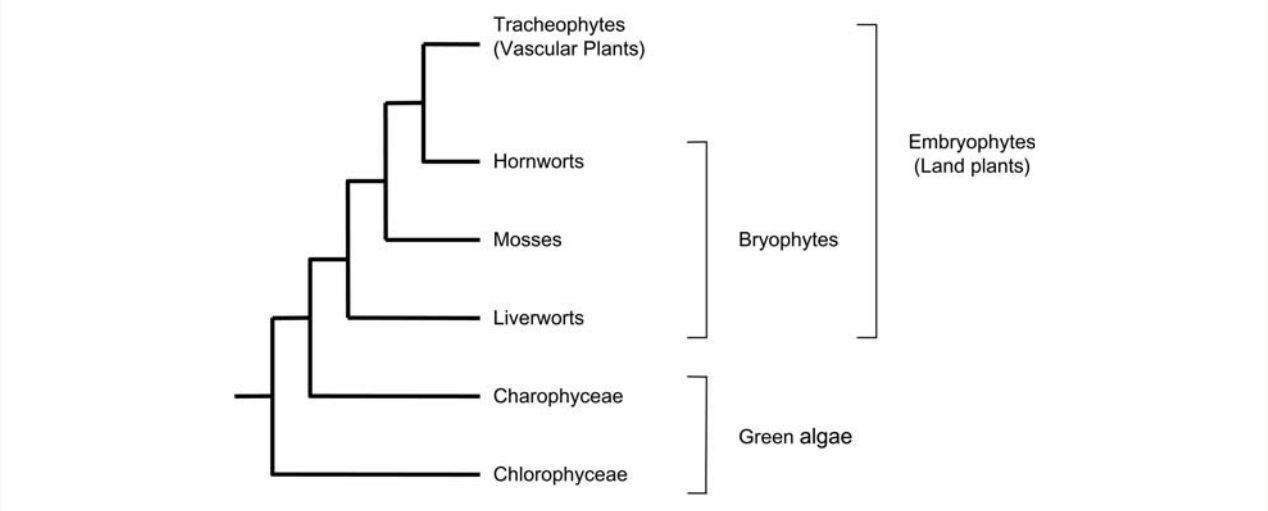

Fig. 1 Phylogenetic tree for land plant evolution derived from analysis by Qui *et al.* (2006). The bryophytes are a paraphyletic group comprising three separate lineages. Together with the vascular plants (which include the angiosperms), bryophytes form the embryophytes, which have a sister group relationship to the green algae.

Development of a durable spore wall is essential for terrestrialization as it enables the spore to withstand physical abrasion, desiccation and UV-B radiation (Wellman 2004). As part of their life cycle, sexually reproducing embryophytes manufacture either spores, or their more derived homologues pollen. The major component of the spore/pollen wall proposed to be of primary importance in enabling resistance to the conditions described above is the highly resistant biopolymer sporopollenin (Ito *et al.* 2007; Cronk 2009).

It seems reasonable to hypothesize that colonization of the land by plants was not possible prior to the evolution of the sporopollenin spore wall, and this adaptation is considered to be a synapomorphy of the embryophytes. Additionally, spore walls are not present in the hypothesized embryophyte antecedents, the green algae (Wellman 2004). However, the production of sporopollenin is highly likely to be pre-adaptive as it is present in a number of different algal groups such as the charophyceans, which have been proposed as the sister group to the embryophytes. In certain charophyceans, sporopollenin occurs, but is located in an inner layer of the zygote wall (Graham 1993).

Phylogenetic studies and fossil evidence have shown that the most basal living land plants are the paraphyletic 'bryophytes' (Kenrick and Crane 1997; Qui *et al.* 2006) (Fig. 1). They comprise the liverworts, mosses and hornworts, and their phylogenetic position should allow us to further elaborate the evolutionary changes that facilitated the conquest of land by plants (Rensing *et al.* 2008). The moss *Physcomitrella patens* is the first 'bryophyte' genome to be sequenced. This genome, through comparisons with angiosperm genomes, is proving to be a valuable tool in experimental studies that attempt to reconstruct genome evolution during the colonization of land (Reski and Cove 2004; Quatrano *et al.* 2007; Rensing *et al.* 2008).

In this review, we first outline the nature of spore/pollen wall development in the major plant groups, before considering emerging understanding of the molecular genetics of pollen wall development. The latter includes identification of genes involved in sporopollenin biosynthesis and exospore formation, callose wall formation and tetrad separation. We also report results from BLAST searches of the basal land plant physcomitrella and the clubmoss *Selaginella moellendorfii* using genes implicated in pollen wall development in arabidopsis.

Spore and pollen wall structure and development

The spore/pollen walls of embryophytes have multiple layers and components that are laid down in a regulated manner during spore/pollen development. Layers containing the macromolecule sporopollenin are the component enabling the resistance of the spore/pollen wall to numerous environmental factors that make life on land challenging. Sporopollenin is highly resistant to physical, chemical and biological degradation procedures. Consequently, its precise chemical composition, structure and biosynthetic route have not yet been ascertained (Meuter-Gerhards *et al.* 1999). Traditional

convention asserts that sporopollenin is a polymer of carotenoid esters (Cronk 2009). However, modern purification, degradation and analytical techniques have shown that it is comprised of polyhydroxylated unbranched aliphatic units with small quantities of oxygenated aromatic rings and phenylpropanoids (Ahlers *et al.* 1999; Domínguez *et al.* 1999).

Modes of sporopollenin deposition in spore and pollen walls

The basic mechanisms involved in the formation of the spore wall, and the deposition of sporopollenin in the exospore/exine, have been illuminated by numerous ultrastructural studies performed on extant and fossil species across the plant kingdom (Paxson-Sowders *et al.* 2001). Blackmore and Barnes (1987) proposed a number of sporopollenin deposition processes apparent in the spore wall. Firstly, they recognized the role of white-line-centred lamellae (WLCL) in this process. The accumulation of sporopollenin on an array of WLCL is regarded as being the most primitive method of sporopollenin deposition and has been identified in a number of algal groups and most, if not all, embryophytes (Wellman 2004). These lamellae materialize at the plasma membrane with sporopollenin polymerizing out onto either side of the white line. They accumulate in a variety of ways to form the spore/pollen wall (Blackmore and Barnes 1987; Blackmore *et al.* 2000; Wellman 2004).

Another mode of exospore/exine formation involves the deposition of sporopollenin from the surrounding cells of the tapetum. Transmission electron microscopy has shown that the tapetal cells possess a highly active secretory system containing lipophilic globules, which are thought to contain the precursors of sporopollenin and are deposited onto the surface either directly contributing to the exospore/exine or forming extra-exosporal layers (Piffanelli *et al.* 1998). Blackmore *et al.* (2000) suggested that a tapetal contribution to the spore wall can take place in a variety of ways, including the addition to the layers formed by the WLCL or directly onto WLCL. Studies of pollen wall formation in angiosperms highlight the role that tapetal cells play in supplying nutrients and lipid components to developing microsporocytes and microspores (Scott *et al.* 1991; Ariizumi *et al.* 2004; de Azevedo Souza *et al.* 2009). Interestingly, the most basal extant land plants (liverworts) lack a tapetum, which is acquired in mosses and vascular plants.

An alternative deposition process involves centripetal accumulation of sporopollenin onto previously formed layers. Blackmore *et al.* (2000) noted that exospore formation may be achieved by sporopollenin accumulation below a pre-existing layer, either by WLCL accumulation or by the deposition of granular or unstructured sporopollenin. A further mode of deposition is observed in seed plants where sporopollenin accumulates within a pre-patterned cell surface glycocalyx referred to as the primexine (Blackmore and Barnes 1987; Blackmore *et al.* 2000; Wellman 2004), which is essentially an exine precursor.

Spore wall development in bryophytes

Spore wall development has been studied in all three of the traditional bryophyte groups (reviewed in Brown and Lemmon 1988, 1990). In the majority of liverworts, immediately after meiosis, a polysaccharide wall (the spore special wall) is laid down outside the plasma membrane (Brown and Lemmon 1985). In many liverworts, this spore special wall seems to function as a primexine in which the pattern of exospore ornamentation is established (Brown and Lemmon 1993). However, in some liverworts exospore ornamentation appears to be determined by exospore precursors produced by the diploid sporocyte prior to meiosis and formation of the haploid spores (Brown *et al.* 1986). The exospore develops centripetally (Brown and Lemmon 1993) based on WLCL formed outside the spore cytoplasm. At completion, the entire exospore comprises sporopollenin deposited on WLCL. At maturity, the lamellate structure thus formed is clearly discernible and is highly characteristic of the liverwort exospore. Liverworts lack a tapetum and there is therefore no input from this source. The innermost layer of fibrillar intine is the final wall layer to be formed (Brown and Lemmon 1993).

Studies of spore wall development in hornworts are limited. As with liverworts, a spore special wall is formed after meiosis and functions as a primexine in which the exospore is set down. It was initially thought that the exospore formed in the absence of WLCL, but Taylor and Renzaglia have recently demonstrated their presence (W.A. Taylor, University of Wisconsin-Eau Claire, USA, pers. comm., 2011). Recent analyses of *Phaeomegaceros fimbriatus* have shown that the mature spore wall has a thin perine-like outer layer, but this represents the remnants of the spore mother cell wall rather than extra-exosporal material derived from a tapetum (Villarreal and Renzaglia 2006).

Three types of spore wall have been recognized in mosses: Bryopsida type, Andreaeidae type and Sphagnidae type (Brown and Lemmon 1990). All three of these types appear to form in the absence of a spore special wall. Bryopsida-type spore walls are homogeneous except for an inconspicuous foundation layer (Fig. 2). This foundation layer forms first via sporopollenin accumulation on WLCL. Subsequently, the homogeneous exospore layer is laid down outside the foundation

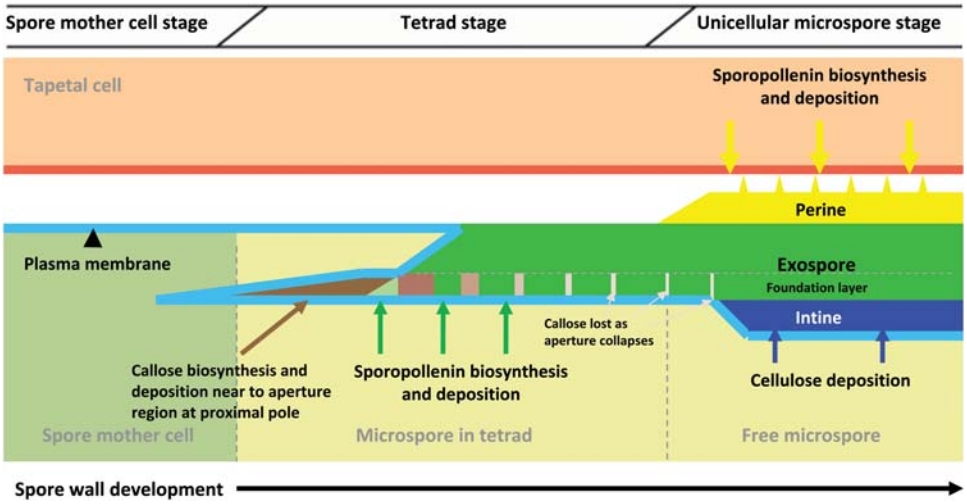

Fig. 2 Proposed model of spore wall development in physcomitrella. The exine foundation layer is laid down first by way of sporopollenin accumulation on WLCL. The rest of the exine layer is deposited outside the foundation layer centrifugally. Note the appearance of callose in the inner exine, which is confined to the expanded aperture region at the proximal pole.

layer in a centrifugal manner. This layer is probably mainly extrasporal in origin. Sometimes additional homogeneous material is also deposited inside the foundation layer. This layer is almost certainly derived from the spore. Following the accumulation of homogeneous material, the spores are coated by an additional extra-exosporal layer, referred to as the perine or perispore, which is derived from the tapetum. Finally, the intine forms.

Spore wall development in the Andreaeidae type is unique among mosses in that they have a spongy exospore that appears to form in the absence of WLCL (Brown and Lemmon 1984). By studying *Andreaea rothii*, Brown and Lemmon (1984) demonstrated that the exospore is instead initiated as discrete homogeneous globules within the coarsely fibrillar network of the spore mother cell. These globules accumulate and form an irregular layer with numerous interstitial spaces. The sequence of spore wall layer development is essentially the same as that of other mosses and the mature wall consists of an inner intine, a spongy exospore and an outer perine (Brown and Lemmon 1984).

Sphagnidae-type moss spore walls are more complex than those of the other mosses and consist of five layers (Brown *et al.* 1982). Unlike other mosses, the exospore of Sphagnidae type comprises two layers: an inner lamellate layer (A-layer) and a thick homogeneous outer layer (B-layer). In addition to the exospore, there is an intine, a unique translucent layer and the outermost perine. The A-layer is the first to form and does so by sporopollenin accumulation on WLCL, and develops evenly around the young spore immediately after meiosis. The homogeneous B-layer is deposited outside the A-layer. Overlying the exospore is a translucent layer that consists of unconsolidated exospore lamellae in a medium of unknown composition. The tapetally derived perine is deposited on top of this unique layer. The study of spore wall development in *Sphagnum lescurii* by Brown *et al.* (1982) suggests that the ontogeny of the wall layers is not strictly centripetal.

Spore wall development in pteridophytes

Spore walls have been investigated in a number of pteridophyte species representing all of the major pteridophyte groups (reviewed in Lugardon 1990; Tryon and Lugardon 1991).

Spore wall development is well understood in the homosporous lycopsid *Lycopodium clavatum* (Uehara and Kurita 1991). Shortly after meiosis, the plasma membrane of the sporogenous cell folds into a pattern that later becomes the reticulate spore sculpture. Small WLCL form on the plasma membrane and accumulate in a centripetal fashion, forming the greater part of the exospore. After the main lamellate part of the exospores is formed, an inner granular layer, possibly derived from the spore cytoplasm, is deposited. In some *Lycopodium* there are no extra-exosporal layers (Uehara and Kurita 1991) whereas in others a thin extra-exosporal layer is deposited after the completion of the exospore (Tryon and Lugardon 1991).

Spore structure and development in heterosporous lycopsids differ between microspores and megaspores.

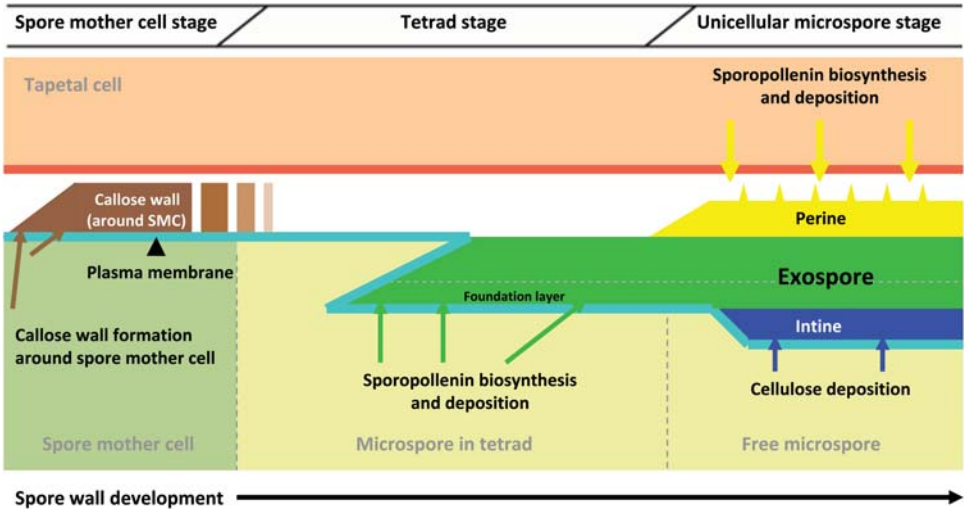

Fig. 3 Proposed model of spore wall development in selaginella microspores. The thin inner exine layer forms first and comprises lamellae formed centripetally on WLCL. The outer exine starts to form once the inner layer is complete. Note the presence of callose at early developmental stages around the spore mother cell.

In the clubmoss selaginella, microspores possess an exospore consisting of two layers (Fig. 3). The thin inner layer is the first to develop and comprises imbricate lamellae that are formed on WLCL in a centripetal direction (Tryon and Lugardon 1991). The outer layer starts to form only once the inner layer is complete. Some selaginella species may also develop a thin perispore or a paraexospore. In the microspores of the heterosporous lycopsid *Isoetes japonica*, a large gap is developed between the two exospore layers (Uehara *et al.* 1991). The outer exospore layer is regarded as a paraexospore as it begins to form before the inner exospore, consists of similar sporopollenin, and is completed at the same time as the inner exospore.

Selaginella megaspore walls contain two layers of similar thickness (Morbelli 1995). The inner and outer layers consist of lamellae and poorly segregated components, respectively. The inner layer does not thicken during exospore development and a dense basal layer is formed by the lamellae. In contrast, the outer layer increases significantly in thickness due to self-assembly (Hemsley *et al.* 1994, 2000; Gabarayeva 2000). During the final stages of sporogenesis, the endospore forms between the plasma membrane and the exospore. In *Isoetes,* the megaspore wall is similar to that of selaginella in terms of development and structure, consisting of two layers, with the formation of the outer layer commencing prior to that of the inner layer. Substantial quantities of silica are deposited within and on top of the outer layer before the exospore is completed. Finally, the endospore is laid down between the plasma membrane and the exospore.

The exospore in homosporous ferns develops centrifugally and is once again bilayered. The inner layer acts as a substructure and consists of varying numbers of fused sheets (extensive interconnected laminae) that form by sporopollenin accumulation on WLCL. The homogeneous outer layer is considerably thicker and contains thin radial fissures and small cavities. An extra-exosporal layer (perispore) forms once the exospore is complete and is deposited from the decaying tapetum. Spore wall development in heterosporous ferns is similar to that observed in homosporous ferns, and is also similar in both microspores and megaspores.

In sphenopsids the spore walls appear to be highly derived (Lugardon 1990), and observations of *Equisetum arvense* have shown that four layers are present in the form of an exospore, an endospore, a middle layer and pseudoelators (Uehara and Kurita 1989). The exospores comprise inner and outer exospores. The broad and homogeneous inner exospore forms first by way of plate-like structures accumulating on the plasma membrane. The outer exospore is then formed by the deposition of granular material on the inner exospore and is similarly wide and homogeneous. Once exospore formation is complete, the middle layer forms in the gap between the exospore and the plasma membrane. The pseudoelators are the next structure to form and consist of two layers. The inner layer comprises longitudinal microfibrils during the early stages of development but eventually becomes homogeneous. The outer layer is also homogeneous and is formed by granules that are released from vesicles in the plasmodial cytoplasm.

The pseudoelators are connected to the spore, by way of the middle layer, at the aperture. The endospore is the final component of the wall to form on the inside of the exospores (Taylor 1986; Uehara and Kurita 1989).

Pollen wall development in gymnosperms

Although differences in pollen wall structure and development are evident in different extant and extinct gymnosperm groups, the main ontogenetic elements appear to be homologous (summarized in Lugardon 1994; Wellman 2009). The pollen mother cell undergoes meiosis to form four haploid microspores. Subsequent development of the exine consists of a number of stages. Firstly, a callose wall forms around the pollen mother cell and subsequently extends around each of the microspores. Next, a matrix develops around each microspore upon which the fibrillar microspore surface coat and later the sexine (consisting of tectum and infratectum components) pattern is established (Zavada and Gabarayeva 1991). The microspore surface coat is deposited between the surface of the microspore and the surrounding tetrad wall prior to the formation of the wall components. This layer is regarded as being equivalent to the primexine in angiosperms. The sexine then begins to form on and within the microspore surface coat. The nexine (inner pollen exine wall) lamina is then formed below this coat; therefore, the sexine is partly developed when the nexine begins to develop. The exine as a whole appears to form in a centripetal direction from the outside inwards (Lugardon 1994). Finally, an intine is deposited on the inside of the pollen exine.

Pollen wall development in angiosperms

Pollen walls in angiosperms typically consist of an outer exine composed of sporopollenin and an inner intine composed of cellulose and pectin (Fig. 4) (Paxson-Sowders *et al.* 1997; Morant *et al.* 2007). Models of development have been proposed based on observations on numerous species, including *Lilium* and arabidopsis (e.g. Suzuki *et al.* 2008). Similar processes have been described in both these species.

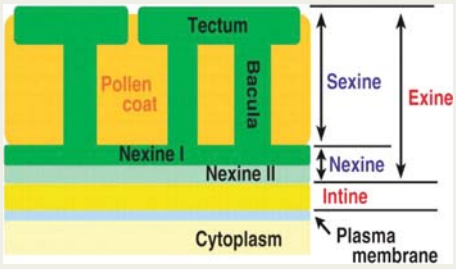

Fig. 4 Diagram of arabidopsis pollen wall structure. The inner intine and the various components of the outer exine are indicated. Note the pollen coat (tryphine and pollenkitt) filling the cavities of the exine sculpture. Taken from Suzuki *et al.* (2008).

Once again, prior to meiosis, the pollen mother cell is surrounded by a callose special cell wall (Blackmore *et al.* 2007). Immediately after meiosis, four microspores derived from the pollen mother cell form a tetrad. A callose special wall surrounds the microspores (Blackmore *et al.* 2007). A cellulose primexine then forms between the plasma membrane and callose wall of each microspore. Both the callose wall and primexine are deposited at the surface of the microspore through processes mediated by the plasma membrane (Blackmore *et al.* 2007). A section of the primexine is then adapted to form column-like structures called the probaculae upon which sporopollenin, secreted by the microspore, will eventually accumulate and polymerize. Sporopollenin deposition and accumulation extend the probaculae, which form the baculae and the tectum (Heslop-Harrison 1963, 1968). The callose wall then degrades and the developing baculae and tectum are exposed to the fluid of the locule and receive sporopollenin secreted by the tapetum. Wall formation is complete when the nexine and intine layers are formed and the primexine recedes and disappears (Suzuki *et al.* 2008). The mature pollen grain is then coated by tryphine and pollenkitt, which are synthesized by the tapetum (Dickinson and Lewis 1973; Blackmore *et al.* 2007).

Summary

While the basic components associated with spore/pollen wall development and structure described above can be localized in different wall regions and influence wall development at different stages across the principal land plant groups, their presence in the majority or all of these groups suggests that their involvement in wall development and sporogenesis as a whole is a signature of embryophytes. The main differences concern the absence, presence and role of callose, and also the mode of sporopollenin deposition.

Callose would appear to play a major role in pollen wall development in angiosperms, where a callose wall surrounds the tetrad and serves as a template for exine development. The role of callose in wall development in other groups is less well defined. In some groups such as pteridophytes, with the exception of selaginella, callose has yet to be identified during sporogenesis and is currently thought to be absent. In some bryophytes (Hepaticopsida: *Anthoceros*, *Geothallus*, *Riccia*; Bryopsida: *Mnium*) (Waterkeyn and Bienfait 1971), callose has been identified around the spore

mother cell but its link to wall development, if any, is not well understood. In this instance, it could be the case that callose is relictual as it is involved in the reproductive systems of some algal groups (Gabarayeva and Hemsley 2006).

Certain modes of sporopollenin deposition are not confined to individual plant groups and different modes have been observed on numerous occasions in a single species. For example, WLCL, while not a constant feature, have been observed in species belonging to all phylogenetic levels of land plants. However, the accumulation of sporopollenin within a pre-patterned microspore surface coat, or primexine, is seemingly confined to gymnosperms and angiosperms.

Molecular genetics of pollen wall development

In recent years there has been a surge in papers describing genes involved in pollen wall development. However, our understanding of the molecular genetics of spore/pollen development remains poor due to the complexity of the developmental process and problems in pinpointing the actual function of the genes involved. Furthermore, research has been confined to particular model angiosperms (Table 1 and Fig. 5), with little or no information on gymnosperm pollen or the spores of 'lower' land plants. This begs the question as to whether similar genes are involved in development of the simple walls of 'lower' land plant spores and the more derived pollen walls of the gymnosperms and angiosperms. However, this research is now beginning to incorporate model plant species from more primitive groups, such as the bryophytes. This extended research will enable the comparison of the molecular genetics of spore/pollen wall development in angiosperms and more primitive plants. The results from this may allow us to assess how conserved are the genes and genetic networks involved in spore/pollen wall development. We begin by reviewing what is known of the molecular genetics of pollen wall development in the angiosperms.

Table 1 Arabidopsis genes implicated in pollen wall development.

Role	Gene	Proposed expression
Sporopollenin biosynthesis and exine formation	*MS2*	Sporophyte
	YRE/WAX2/FLP1	Sporophyte
	CYP703A2	Sporophyte and microspores
	CYP704B1	Sporophyte
	ACOS5	Sporophyte
	RPG1	Sporophyte and microspores
	NEF1	Sporophyte
	KNS5-10	Sporophyte
	KNS4	Sporophyte
	ABCG26	Sporophyte and microspores
	LAP5/PKSB	Sporophyte
	LAP6/PKSA	Sporophyte
	TKPR1/DRL1	Sporophyte
	TKPR2/CCRL6	Sporophyte
	AtMYB103/MS188 (TF)	Sporophyte
	MS1 (TF)	Sporophyte
	AtbZIP34 (TF)	Sporophyte and gametophyte
Exine formation (probaculae)	*DEX1*	Unknown
	TDE1/DET2	Unknown
	KNS2, 3, 12	Sporophyte
Intine formation	*FLA3*	Sporophyte and microspores
Callose wall formation	*CALS5/LAP1*	Unknown
	KNS1, 11	Sporophyte
Tetrad separation	*QRT1-3*	Sporophyte
	A6	Sporophyte

TF denotes transcription factor.

Arabidopsis genes implicated in sporopollenin biosynthesis and exine formation

A number of arabidopsis genes associated with the biosynthesis of exine encode proteins with sequence homology to enzymes involved in fatty acid metabolism (Dobritsa *et al.* 2009). Aarts *et al.* (1997) observed expression of the *MALE STERILITY 2* (*MS2*) gene in the tapetum of wild-type plants at, and shortly after, the release of microspores from tetrads and noted that *MS2* mutants produced pollen grains that lacked an exine layer. The exine layer had been replaced by a thin layer of unknown composition. *MS2* encodes a protein with sequence similarity to long-chain fatty acyl reductases, and expression of the MS2 protein in bacteria leads to the increased synthesis of fatty alcohols (Doan *et al.* 2009). Taken together, these data suggest that an *MS2*-linked enzymatic pathway is required for the synthesis of sporopollenin (Aarts *et al.* 1997; Ariizumi *et al.* 2008; Dobritsa *et al.* 2009).

Another gene implicated in exine formation is *YORE-YORE* (*YRE*)/*WAX2*/*FACELESS POLLEN1* (*FLP1*). Ariizumi *et al.* (2003) suggested that this gene encodes a transporter or catalytic enzyme that is involved in

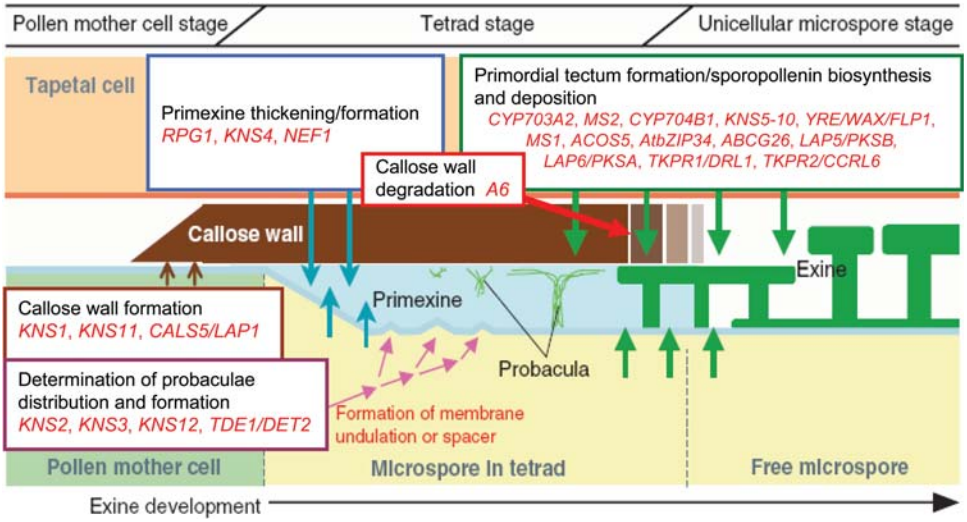

Fig. 5 Proposed functions of genes implicated in arabidopsis pollen wall exine development. Expected time and location of gene expression are indicated. Not all arabidopsis genes described in this review are included due to a lack of information regarding the time and locality of their expression. Modified from Suzuki *et al.* (2008).

wax synthesis in stems and siliques, in the tryphine and in sporopollenin synthesis. As with *MS2*, the pollen exine in *YRE/FLP1* mutants is poorly constructed and easily damaged, suggestive of defective sporopollenin. Expression analyses in the same study suggest that *FLP1* is expressed in the tapetum, which is supported by the fact that the *FLP1* mutant phenotype is sporophytically controlled (Ariizumi *et al.* 2003). In addition, Rowland *et al.* (2007) demonstrated that the *ECERIFIUM 3* (*CER3*) gene encodes a protein of unknown function identical to *YRE/WAX2/FLP1* and is therefore allelic to *YRE/WAX2/FLP1*.

Morant *et al.* (2007) showed that the arabidopsis cytochrome P450 enzyme *CYP703A2* is also necessary for the synthesis of sporopollenin. The *CYP703* cytochrome P450 family is specific to embryophytes and each plant species contains a single *CYP703* (Morant *et al.* 2007). The exine layer in *CYP703A2* knock-out mutants is significantly underdeveloped. Sporopollenin also appeared to be absent as the fluorescent layer around the pollen associated with the presence of phenylpropanoid units in sporopollenin was absent in *CYP703A2* mutant plants (Morant *et al.* 2007). Morant *et al.* (2007) demonstrated that lauric acid and in-chain hydroxy lauric acids are present in the plant substrate and product for this enzyme. These are important building blocks in the synthesis of sporopollenin and facilitate the formation of ester and ether linkages with phenylpropanoid units. Furthermore, the same study showed that *CYP703A2* is expressed in the anthers of developing arabidopsis flowers with initial expression detectable at the tetrad stage in the microspores and the tapetum (Morant *et al.* 2007), consistent with a role in exine formation.

Dobritsa *et al.* (2009) described another cytochrome P450, *CYP704B1*, and demonstrated that this gene is essential for exine development. *CYP704B1* mutants produce pollen walls that lack a normal exine layer. The exine layer was replaced with a thin layer of material and irregular distribution of aggregates that may have been sporopollenin. The pollen walls also exhibited a characteristic striped surface, unlike the reticulate pattern displayed by the wild type, to which Dobritsa *et al.* (2009) designated the name *zebra* phenotype. It has also been shown that heterologous expression of *CYP704B1* in yeast catalyses ω-hydroxylation of long-chain fatty acids, consistent with a role in sporopollenin synthesis (Dobritsa *et al.* 2009). Dobritsa *et al.* (2009) have suggested that these ω-hydroxylated fatty acids, in concert with the formation of in-chain hydroxylated lauric acids catalysed by *CYP703A2*, may serve as vital monomeric aliphatic building blocks in the formation of sporopollenin. Analyses of the genetic relationships between *CYP704B1*, *CYP703A2* and *MS2* (which as described above encodes a fatty acyl reductase) along with expression analyses and observation of similar *zebra* phenotypes in all three mutants indicate that these genes are involved in the same pathway within the sporopollenin synthesis framework and are co-expressed (Dobritsa *et al.* 2009). In addition, an orthologue of *CYP704B1* (*BnCYP704B1)* has recently

been identified in *Brassica napus*, and mutants in this gene exhibit defective exine layers (Yi *et al.* 2010).

Another gene reported to participate in exine formation, *ACOS5*, has recently been described (de Azevedo Souza *et al.* 2009). This encodes a fatty acyl-CoA synthetase with broad *in vitro* preference for the medium-chain fatty acids required in tapetal cells for sporopollenin monomer synthesis. Mutations in *ACOS5* significantly compromise the development of the pollen wall, which appears to lack sporopollenin and exine. The defect in pollen formation in *ACOS5* mutants coincides with the deposition of exine at the unicellular microspore stage (de Azevedo Souza *et al.* 2009). Additionally, after analyses of *ACOS5* expression in developing anthers, de Azevedo Souza *et al.* (2009) proposed that it is also involved in the same biochemical pathway as the *CYP703A2*, *CYP704B1* and *MS2* genes.

The *RUPTURED POLLEN GRAIN1* (*RPG1*) gene, which encodes a plasma membrane protein, and the *NO EXINE FORMATION1* (*NEF1*) gene, which encodes a plastid integral membrane protein, are both required for primexine development (Ariizumi *et al.* 2004; Guan *et al.* 2008). Guan *et al.* (2008) revealed that exine pattern formation in *RPG1* mutants is defective as sporopollenin is randomly distributed over the surface of the pollen grain. Primexine formation of microspores in *RPG1* mutants is abnormal at the tetrad stage, which results in imperfect deposition of sporopollenin on the microspores (Guan *et al.* 2008). *RPG1* plants experience microspore rupture and cytoplasmic leakage, suggesting that cell integrity had been impaired in the microspores. The same study demonstrated that *RPG1* is strongly expressed in the tapetum and the microspores during male meiosis (Guan *et al.* 2008). Ariizumi *et al.* (2004) showed that *NEF1* mutants exhibited similarly defective primexine and that although sporopollenin was present it was not deposited onto the plasma membrane of the microspore because of the lack of normal primexine. Ariizumi *et al.* (2004) tentatively suggest that *NEF1* is expressed in the tapetum and is sporophytically controlled. Additionally, it was proposed that *NEF1* is likely to be involved in exine formation at earlier developmental stages than other exine formation genes, such as *MS2* and *FLP1*, since the exine is more poorly developed in *NEF1* plants (Ariizumi *et al.* 2004).

Suzuki *et al.* (2008) also identified a number of genes involved in the construction of exine and pollen development in general. They managed to successfully isolate 12 *KOANASHI* mutants (*KNS1*–*KNS12*), which were found to be recessive and thus likely to affect pollen development sporophytically. The 12 mutants were categorized into four types. Type 3 (*KNS5*–*KNS10*) mutants displayed abnormal tectum formation on the pollen surface, and these genes therefore appear to be required either for creating primordial tectum (onto which sporopollenin is deposited) in the space between the primexine and the callose wall, or for depositing sporopollenin itself (Suzuki *et al.* 2008). Additionally, the type 2 mutant (*KNS4*) exhibits a thin exine layer mostly due to shortened baculae. It is proposed that baculae extension is closely linked to the thickening of primexine; therefore, *KNS4* is likely to be a novel gene that regulates the thickening of the primexine layer (Suzuki *et al.* 2008).

Recently, Quilichini *et al.* (2010) proposed that *ATP-BINDING CASSETTE G26* (*ABCG26*) plays a crucial role in exine formation. *Abcg26-1* mutants lack an exine layer, and expression studies showed that *ABCG26* is transiently and locally expressed in the tapetum post meiosis. Quilichini *et al.* (2010) suggest that *ABCG26* transports sporopollenin precursors across the tapetum plasma membrane to the anther locule for polymerization on the surface of the developing microspores.

Other genes that have recently been associated with a defective exine include *LESS ADHESIVE POLLEN 5/POLYKETIDE SYNTHASE B* (*LAP5/PKSB*) and *LESS ADHESIVE SYNTHASE 6/POLYKETIDE SYNTHASE A* (*LAP6/PKSA*), which are also specifically and transiently expressed in the tapetum during microspore development (Kim *et al.* 2010). Mutant plants compromised in the expression of *LAP5/PKSB* and *LAP6/PKSA* exhibited significantly defective exine layers, and a double *LAP5/PKSB LAP6/PKSA* mutant appeared to completely lack an exine layer. These two genes are co-expressed with *ACOS5,* and recombinant *LAP5/PKSB* and *LAP6/PKSA* proteins were able to generate tri- and tetraketide alpha-pyrone compounds *in vitro* from a wide range of potential *ACOS5*-generated fatty acyl-CoA starter substrates via condensation with malonyl-CoA. These compounds would therefore appear to be required for sporopollenin biosynthesis (Kim *et al.* 2010). Additionally, two closely related genes, *TETRAKETIDE alpha-PYRONE REDUCTASE1* (*TKPR1/DRL1*) and *2* (*TKPR2/CCRL6*), encode oxidoreductases, which have been found to be active on the tetraketide products produced by *LAP5/PKSB* and *LAP6/PKSA*. TKPR activity reduces the carbonyl function of the tetraketide alpha-pyrone compounds synthesized by *LAP5/PKSB* and *LAP6/PKSA*, and together with the activities associated with *LAP5/PKSB*, *LAP6/PKSA* and *ACOS5*, forms a biosynthetic pathway that ultimately produces hydroxylated alpha-pyrone compounds, potential precursors for sporopollenin (Grienenberger *et al.* 2010).

Arabidopsis transcription factors involved in sporopollenin and exine formation

A number of transcription factors participating in the development of exine have been described. *AtMYB103/MS188* is a MYB transcription factor that is specifically

expressed in the anthers and trichomes of arabidopsis (Li *et al.* 1999; Higginson *et al.* 2003). Zhang *et al.* (2007) have shown that *AtMYB103/MS188* directly regulates the expression of the previously described exine formation gene *MS2* and the callase-related *A6* gene. Knock-out mutants of *AtMYB103/MS188* resulted in early tapetal degeneration and abnormal microspores. Additionally, expression of the *MS2* gene was not detected in the anthers of the *AtMYB103/MS188* mutants (Zhang *et al.* 2007). The *MALE STERILITY1 (MS1)/HACKLY MICROSPORE (HKM)* gene, encoding a leucine zipper-like, PHD-finger motif transcription factor, is also involved in tapetum function (Ariizumi *et al.* 2005; Vizcay-Barrena and Wilson 2006; Ito *et al.* 2007; Yang *et al.* 2007). Phenotypic analysis of *MS1* mutants by Ito *et al.* (2007) indicated that *MS1* is required for transcriptional regulation of genes involved in primexine formation, sporopollenin synthesis and tapetum development. Lack of *MS1* expression results in changes in tapetal secretion and exine structure with the appearance of autophagic vacuoles and mitochondrial swelling, suggesting that the tapetum is broken down by necrosis rather than by apoptosis as observed in the wild type (Vizcay-Barrena and Wilson 2006; Yang *et al.* 2007). Yang *et al.* (2007) further demonstrated that *MS1* is expressed in the tapetal cells in a developmentally regulated manner between the late tetraspore stage and microspore release.

Another transcription factor involved in exine formation has been identified by Gibalová *et al.* (2009), who demonstrated that *AtbZIP34* mutants exhibit defects in exine structure. The exine layer is wrinkled, and the baculae and tecta are deformed. Additionally, 50% of mutant pollen exhibited a wrinkled intine layer. Despite these abnormalities, high levels of pollen abortion or male sterility were not observed (Gibalová *et al.* 2009). Transcriptomic analyses revealed that expression of the proposed primexine development gene, *RPG1*, is significantly downregulated in *AtbZIP34* mutant pollen. Given the expression profiles of both genes, it is possible that *RPG1* expression is regulated by *AtbZIP34* (Gibalová *et al.* 2009). Analyses also suggested sporophytic and gametophytic roles for *AtbZIP34* in exine and intine formation.

The observation that many of the genes described in the previous sections are predominately expressed sporophytically is somewhat at odds with the fact that exine development mostly occurs at the surface of individual microspores after meiosis. Suzuki *et al.* (2008) propose that this apparent contradiction may possibly be explained by many of these genes being expressed in pollen mother cells so that the relevant mRNA or proteins are inherited by the derived microspores.

Arabidopsis genes associated with probaculae formation

At present, five arabidopsis genes have been specifically associated with the formation of probaculae, which is an important component in the exine development process. The *DEFECTIVE IN EXINE1* (*DEX1*) gene encodes a novel membrane protein that is required for anchoring sporopollenin to the surface of the microspores and is implicated in probacula formation (Paxson-Sowders *et al.* 1997, 2001). Sporopollenin synthesis still takes place in *DEX1* mutants but primexine development is delayed and ultimately reduced, which alters membrane formation and therefore the deposition of sporopollenin. Spacers do not form in the primexine, which results in sporopollenin being randomly deposited on the plasma membrane (Paxson-Sowders *et al.* 2001). Additionally, sporopollenin does not appear to be anchored to the microspore and forms bulky aggregates on the developing microspore and locule walls, and the pollen wall does not form, which results in pollen degradation (Paxson-Sowders *et al.* 2001).

Ariizumi *et al.* (2008) suggested that the *TRANSIENT DEFECTIVE EXINE1* (*TDE1*)/*DE-ETIOLATED2* (*DET2*) gene is also involved in probacula development. Specifically, they proposed that *TDE1*/*DET2* is involved in brassinosteroid synthesis, a hormone purported to control the rate or efficiency of the initial process of exine formation. Primexine synthesis is defective in *TDE1*/*DET2* mutant plants which ultimately fail to produce probacula at the tetrad stage (Ariizumi *et al.* 2008). Additionally, globular sporopollenin is haphazardly deposited onto the microspore at the early uninucleate microspore stage (Ariizumi *et al.* 2008). As with *DEX1* mutants, sporopollenin apparently failed to anchor to the plasma membrane of the microspore and instead aggregated on the locule wall and in the locule at the uninucleate microspore stage (Paxson-Sowders *et al.* 2001; Ariizumi *et al.* 2008). However, despite these defects, reticulate exine was clearly formed at the later stage in *TDE1*/*DET2* mutants, which is in contrast to other mutants displaying primexine defects, such as *DEX1*, which always fail to produce normal exine at the later stages. This suggests that mutations in *TDE1*/*DET2* do not result in defects at critical stages of exine development (Ariizumi *et al.* 2008). Expression analysis also demonstrated that brassinosteroids may be synthesized in developing microspores. The same analysis also showed that *TDE1*/*DET2* mutations did not affect the expression of genes implicated in exine development. This suggests that brassinosteroids support exine development in a distinct pathway (Ariizumi *et al.* 2008).

The *KNS2*, *3* and *12* genes, designated type 4 genes by Suzuki *et al.* (2008), have also been associated with probacula formation. Type 4 mutants were shown to exhibit abnormal positioning of baculae, which were densely distributed. This suggests that the type 4 genes govern the position of probacula formation either by forming undulations on the microspore plasma membrane at the tetrad stage or by forming spacers (Suzuki *et al.* 2008). Additionally, Suzuki *et al.* (2008), using map-based cloning, were able to reveal that one of the type 4 genes, *KNS2*, encodes sucrose phosphate synthase, which is proposed to be potentially involved in primexine synthesis or callose wall formation, which are known to be important for the positioning of probaculae. Further studies are required to specifically determine the time and location of expression of *KNS* type 4 genes.

Arabidopsis genes connected to intine formation

Recently, Li *et al.* (2010) have proposed that the fasciclin-like arabinogalactan protein gene *FLA3* is involved in the development of the intine layer by playing a role in the deposition of cellulose. The down-regulation of *FLA3* via RNAi results in the appearance of a thinning intine layer and the production of ~50% non-viable pollen grains, many of which display a wrinkled or shrunken phenotype. Expression studies showed that *FLA3* is specifically expressed in pollen tubes and pollen grains, and is localized to the cell membrane (Li *et al.* 2010). Other arabidopsis genes have also been implicated in intine formation, including the reversibly glycosylated peptide genes, *RGP1* and *RGP2*. Pollen grains in double-knock-out plants of *RGP1* and *RGP2* exhibit unusually enlarged vacuoles and a poorly defined intine layer (Drakakaki *et al.* 2006).

Arabidopsis genes implicated in callose wall formation

To date, three arabidopsis genes have been associated with callose wall formation. Dong *et al.* (2005) and Nishikawa *et al.* (2005) have demonstrated that the *CALLOSE SYNTHASES (CALS5)/LESS ADHERENT POLLEN (LAP1)* gene encodes a callose synthase essential for callose wall formation. *CALS5/LAP1* mutants lack callose on the cell wall of pollen mother cells, tetrads and microspores, which ultimately results in the development of sterile pollen due to the degeneration of microspores (Dong *et al.* 2005). Additionally, exine structure in the mutant plants was severely deformed, affecting the baculae and tecta structure, and tryphine was haphazardly deposited as globular structures (Dong *et al.* 2005). This implies that the callose wall is vitally important for the formation of a properly sculpted exine (Dong *et al.* 2005). Expression analyses have produced varied results with regard to *CALS5/LAP1* and suggest that the gene is expressed in either pollen mother cells or pollen tetrads, or possibly both cell types (Nishikawa *et al.* 2005).

The *KNS1* and *KNS11* genes constitute the type 1 genes as described and classified by Suzuki *et al.* (2008). Type 1 mutant plants exhibit pollen grains that display a highly collapsed exine structure in which the tecta disappear and the baculae deform into globular protrusions. Additionally, mature pollen grains of both genes were reduced in size and in number, and were distorted in shape (Suzuki *et al.* 2008). This phenotype closely resembled the pollen phenotype of *CALS5/LAP1* mutants described above (Dong *et al.* 2005; Nishikawa *et al.* 2005; Suzuki *et al.* 2008). This resemblance, along with the recessive nature of the type 1 genes, suggests that *KNS1* and *KNS11* are expressed in pollen mother cells and are important in synthesizing or secreting callose (Suzuki *et al.* 2008).

Arabidopsis genes involved in tetrad separation

The *QUARTET* (*QRT*) genes have been identified as being required for pollen separation during normal pollen development (Preuss *et al.* 1994; Rhee and Somerville 1998; Francis *et al.* 2006). In wild-type arabidopsis pollen, degradation of the pollen mother cell walls takes place, which releases the individual microspores as single pollen grains (Francis *et al.* 2006). Mutations in any of the *QRT1*, *QRT2* or *QRT3* genes cause the outer walls of the microspores to become fused following meiosis, resulting in pollen grains being released as tetrads (Preuss *et al.* 1994; Rhee and Somerville 1998; Francis *et al.* 2006). Rhee and Somerville (1998) have demonstrated that the enzymatic removal of callose at the tetrad stage is not sufficient to release the microspores. In *QRT1* and *QRT2* mutants, pectic components were detectable at the time of tetrad separation, which was not the case in the wild type. This suggests that the persistence of pectin in the pollen mother cell wall is associated with tetrad separation failure (Rhee and Somerville 1998).

Pollen mother cell primary cell walls have been proposed to play a significant part in cell–cell adhesion mechanisms (Rhee and Somerville 1998). The pectins of the primary cell wall have been shown to consist mostly of homogalacturan, a polymer of β-1,4-galacturonic acid (GalUA), rhamnogalacturonan I and rhamnogalacturonan II (branching polymers of GalUA, Ara and Rha) (Brett and Waldron 1996; Tucker and Seymour 2002). As pectin is synthesized, the backbone of GalUA is in a methylesterified state that can then be demethylesterified by pectin methylesterases and cleaved by endo-polygalacturonases, which results in loosening of the cell wall (Schols and Voragen 2002;

Francis *et al.* 2006). *QRT1* and *QRT2* have been proposed to encode pectin methylesterases (Francis *et al.* 2006). Expression analysis has shown that *QRT1* is expressed shortly after meiosis is complete (Francis *et al.* 2006). Additionally, Rhee *et al.* (2003) have identified *QRT3* as being an endopolygalacturonase that degrades the pectic polysaccharides of pollen mother cells. It has been demonstrated that the *QRT3* gene is specifically and transiently expressed in tapetal cells during microspore release from the tetrad (Rhee *et al.* 2003). Immunohistochemical localization of *QRT3* suggests that the protein it encodes is secreted from the tapetum during the early stages of microspore development (Rhee *et al.* 2003).

Genes associated with callose wall degradation have, to date, not been definitively identified. Frankel *et al.* (1969) and Stieglitz and Stern (1973) demonstrated that the tetrad callose wall is degraded by β-1,3-glucanase activity secreted from the tapetal cells. While a number of candidate β-1,3-glucanase-encoding genes have been identified, none has been confirmed as a callase (Hird *et al.* 1993). However, Hird *et al.* (1993) have proposed that the *A6* gene may encode a component of the callase enzyme complex due to the fact that it is tapetum specific, has a strong sequence similarity to other β-1,3-glucanases, and is temporally expressed at peak levels when the plant normally expresses callase. Future identification of *A6* mutant plants is needed to confirm the gene as a callase. Additionally, real-time reverse transcriptase–polymerase chain reaction analysis conducted by Zhang *et al.* (2007) has suggested that *A6* is regulated by the *AtMYB103/MS188* gene.

Summary

The genes and associated mutants described above have thus far provided clues with regard to wall development in arabidopsis, particularly with respect to exine formation and sporopollenin biosynthesis. They suggest that wall development is controlled by both the diploid sporophyte and haploid microspores, and have identified the sporophytic tapetum, in addition to the microspores themselves, as an important site for sporopollenin biosynthesis. However, large gaps in our understanding remain regarding the genetic network and biosynthetic route responsible for the formation of the pollen wall.

Flowering plant homologue genes for spore wall development are present in the moss *P. patens* and the lycophyte *S. moellendorfii*

Research into the molecular genetics of spore wall development in basal plants has thus far been extremely limited. Schuette *et al.* (2009), using immuno-light and immuno-electron microscopy, identified the presence of callose in the spores of physcomitrella where it was deposited in the inner exospore layer near the expanded aperture region (local expansion of the intine layer) at the proximal pole, suggesting that callose is involved in aperture expansion during wall development (Fig. 2). It is proposed that a *CALS5* homologue is present in the physcomitrella genome and is involved in spore wall development (Schuette *et al.* 2009). However, expression studies of the *CALS5* homologue, required to further address this proposition, have yet to be undertaken in physcomitrella.

In addition to *CALS5*, the majority of the other arabidopsis genes described in this review have been annotated and their protein sequences are available on The Arabidopsis Information Resource (TAIR) website (Website 1). We have used these protein sequences to search for homologous genes in the physcomitrella (Website 2) and selaginella genomes (Websites 3 and 4). The results suggest that homologues of all known arabidopsis pollen wall-associated genes are present in the physcomitrella genome, with the number of proposed homologues ranging from one for *DEX1* and *TDE1/DET2* to in excess of 50 for *CYP703A2* and *AtMYB103/MS188*. Similar results are observed in selaginella with homologues of all but one (*QRT3*) of the arabidopsis pollen wall-associated genes present in its genome, ranging from two homologues in *DEX1*, *MS1* and *NEF1* to more than 50 once again for *CYP703A2*, *AtMYB103/MS188* and the callase-related *A6* gene (Table 2).

These results indicate that the vast majority of the pollen wall-associated genes belong to multigene families, and therefore, as with almost every developmental, signalling and metabolic context (Kafri *et al.* 2009), there is a high potential for genetic redundancy. Analyses of large collections of expressed sequence tag sequences have suggested that physcomitrella is a palaeopolyploid with a whole-genome duplication having occurred between 30 and 60 million years ago (Rensing *et al.* 2007), which may account for the presence of some of these gene duplications. While the selaginella sequence data do not indicate any ancient whole-genome duplication (Banks *et al.* 2011), the results here show that for most of the pollen wall-associated genes numerous copies (greater in number than in the physcomitrella genome) are also present in the selaginella genome. This suggests the occurrence of many small-scale duplication events and a greater level of gene redundancy and/or number of pseudogenes in selaginella compared with physcomitrella.

Table 2 TBLASTN results of searches of the genomes of physcomitrella and selaginella with arabidopsis pollen wall genes. An e-value threshold of $1e^{-4}$ was used as an initial filter to determine number of homologues. Identity percentages (≥30%) and BLAST scores were then used to filter numbers further. Best match is defined as BLAST hit with the highest BLAST score.

General function in arabidopsis	Arabidopsis gene	Gene reference	Proposed gene class	No. of proposed homologues in physcomitrella	% identity of best match in physcomitrella	BLAST score (bits) of best match in physcomitrella	No. of proposed homologues in selaginella	% identity of best match in selaginella	BLAST score (bits) of best match in selaginella
Sporopollenin biosynthesis and exine formation	*MS2*	AT3G11980	Fatty acyl reductase	2	48	1293	4	48	1221
	YRE/WAX2/FLP1/CER3	AT5G57800	Aldehyde decarbonylase	4	47	1528	9	49	1642
	CYP703A2	AT1G01280	Cytochrome P450	31	46	1106	>50	46	1218
	CYP704B1	AT1G69500	Cytochrome P450	8	57	1612	27	60	1514
	ACOS5	AT1G62940	Fatty acyl-CoA synthetase	11	50	1378	26	51	1401
	RPG1	AT5G40260	Unknown plasma membrane protein	6	37	392	26	43	439
	NEF1	AT5G13390	Unknown plastid integral membrane protein	1	44	2487	2	41	2168
	KNS5-10 (type 3)	–	Unknown	–	–	–	–	–	–
	KNS4 (type 2)	–	Unknown	–	–	–	–	–	–
	ABCG26	AT3G13220	ATP-binding cassette transporter	3	46	1420	6	52	1593
	LAP5/PKSB	AT4G34850	Polyketide synthase	21	57	1194	9	59	1149
	LAP6/PKSA	AT1G02050	Polyketide synthase	16	50	944	9	47	932
	TKPR1/DRL1	AT4G35420	Oxidoreductase	7	51	868	32	52	850
	TKPR2/CCRL6	AT1G68540	Oxidoreductase	7	52	881	31	52	879
	AtMYB103/MS188	AT5G56110	R2R3 MYB transcription factor	>50	75	587	>50	66	482
	MS1	AT5G22260	PHD-type transcription factor	2	37	1109	2	38	1124
	AtbZIP34	AT2G 42380	bZIP transcription factor	13	60	315	9	67	319
Exine formation (probaculae)	*DEX1*	AT3G09090	Unknown membrane protein	1	70	1663	2	50	2210
	TDE1/DET2	AT2G38050	Unknown	1	40	455	4	43	495
	KNS2, 3, 12 (type 4)	AT5G11110 (KNS2)	Sucrose phosphate synthase (KNS2)	2 (KNS2)	52 (KNS2)	2839 (KNS2)	4	53	2809

Continued

Table 2 *Continued*

General function in arabidopsis	Arabidopsis gene	Gene reference	Proposed gene class	No. of proposed homologues in physcomitrella	% identity of best match in physcomitrella	BLAST score (bits) of best match in physcomitrella	No. of proposed homologues in selaginella	% identity of best match in selaginella	BLAST score (bits) of best match in selaginella
Intine formation	*FLA3*	AT2G24450	Fasciclin-like arabinogalactan	0	n/a	n/a	5	33	221
Callose wall formation	*CALS5/LAP1*	AT2G13680	Callose synthase	12	64	6575	13	64	6597
	KNS1,11 (type 1)	–	Unknown	–	–	–	–	–	–
Tetrad separation	*QRT1*	AT5G55590	Pectin methylesterase	45	47	804	45	54	821
	QRT2	AT3G07970	Pectin methylesterase	3	41	733	20	44	823
	QRT3	AT4G20050	Endopolygalcturonase	2	50	996	0	n/a	n/a
	A6	CAA49853	Callase	31	43	982	>50	45	980

Conclusions and forward look

The presence of pollen wall-associated genes in physcomitrella and selaginella provides a strong incentive for further study in this area. Survival in a terrestrial environment has been proposed to involve the evolutionary acquisition of a number of traits, including a specialized spore wall (Wellman 2004; Cronk 2009). As described above, the morphology of moss and lycopsid spores is markedly simpler yet bears similarities to that of 'higher' plant pollen. Can the development of a specialized spore wall be traced to the recruitment of a few key gene products for a novel function, or did it involve a more gradual accretion of cell wall constituents in novel architectures? To what extent were the gametophyte and sporophyte involved in the deposition of the spore wall in early evolutionary history, and can this be inferred from the study of extant bryophytes and other branches of the plant evolutionary tree? The finding that homologues of angiosperm pollen cell wall-associated genes are easily identifiable in extant bryophytes and lycopsids opens the door to functional analyses of these genes. Of course, the potentially high levels of redundancy observed in physcomitrella and selaginella present challenges to a functional approach, but expression studies will help direct future research in this area and circumvent some of these difficulties. The use of high-throughput sequencing strategies and/or microarray approaches will allow researchers to identify homologues that might potentially play a role in spore wall biogenesis, and the development of techniques for gene knock-outs and gene swap experiments will allow testing of hypotheses on the conservation of gene function in the spore wall, as has already been achieved in the area of leaf, root and stomata EvoDevo studies (Harrison *et al.* 2005; Menand *et al.* 2007; Chater *et al.* 2011; Ruszala *et al.* 2011). These studies strongly suggest that true spore/pollen wall gene homologues are likely to exist in lower land plants, particularly those genes associated with wall structures that are present across embryophytes. It is also reasonable to suggest that genes associated with more specialist wall elements, such as the primexine which is only present in higher land plant groups, are less well conserved.

Although a focus in this area has been on bryophytes, such as physcomitrella (due to the availability of appropriate genetic resources), the development of novel experimental systems, such as selaginella and the liverwort *Marchantia polymorpha*, will allow a deeper insight into spore evolution and, more broadly, enable us to better assess whether the key mechanisms required for plant terrestrialization have been conserved over 400 million years of land plant evolution.

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Contributions by the authors

S.W. made the greatest contribution to this work and is therefore first author.

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Conflicts of interest statement

None declared.

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Appendix

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