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ORIGINAL ARTICLE

Developmental origins of structural diversity in pollen walls of Compositae

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Abstract Compositae exhibit some of the most complex and diverse pollen grains in flowering plants. This paper reviews the evolutionary and developmental origins of this diversity in pollen structure using recent models based on the behaviour of colloids and formation of micelles in the differentiating microspore glycocalyx and primexine. The developmental model is consistent with observations of structures recovered by pollen wall dissolution. Pollen wall diversity in Compositae is inferred to result from small changes in the glycocalyx, for example ionic concentration, which trigger the self-assembly of highly diverse structures. Whilst the fine details of exine substructure are, therefore, not under direct genetic control, it is likely that genes establish differences in the glycocalyx which define the conditions for self-assembly. Because the processes described here for Compositae can account for some of the most complex exine structures known, it is likely that they also operate in pollen walls with much simpler organisation.

This paper is dedicated to the memory of Donald Claugher.

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Introduction

Compositae is not only the largest family of flowering plants (Funk et al. 2005), but is also remarkable for the great diversity of internal and external forms encountered in its pollen grains (Figs. 1, 2). Many investigators have made observations on Compositae pollen, ever since the invention of microscopy (for a comprehensive bibliography see Wortley et al. 2009) but the details were first documented systematically, using light microscopy, by Wodehouse (1935), Erdtman (1952), Wagenitz (1955, 1976) and Stix (1960). These researchers revealed a high degree of variation in both surface ornamentation, which ranges from psilate (not shown) to echinate (e.g. Fig. 1e), lophate (Fig. 1i) and echinolophate (Fig. 1g), and internal structure (Figs. 1, 2), variation which has great potential in helping to elucidate evolutionary relationships in Compositae (Blackmore et al. 2009).

The advent of electron microscopy enabled Skvarla and co-workers (Skvarla and Larson 1965; Skvarla and Turner 1966; Tomb et al. 1974; Skvarla et al. 1977) to describe the major patterns of exine structure in the Compositae in detail and to establish a number of exine types which subsequently became adopted widely in palynological and systematic studies. The Anthemoid exine type (Skvarla et al. 1977) is characterised by solid columellae which connect to the foot layer without interruption by a cavea (Figs. 1b, d, f, l, 2a–e, g, i, k, m; Blackmore et al. 1984). In the Helianthoid exine type (Skvarla et al. 1977) the columellae contain internal foramina, visible under TEM,

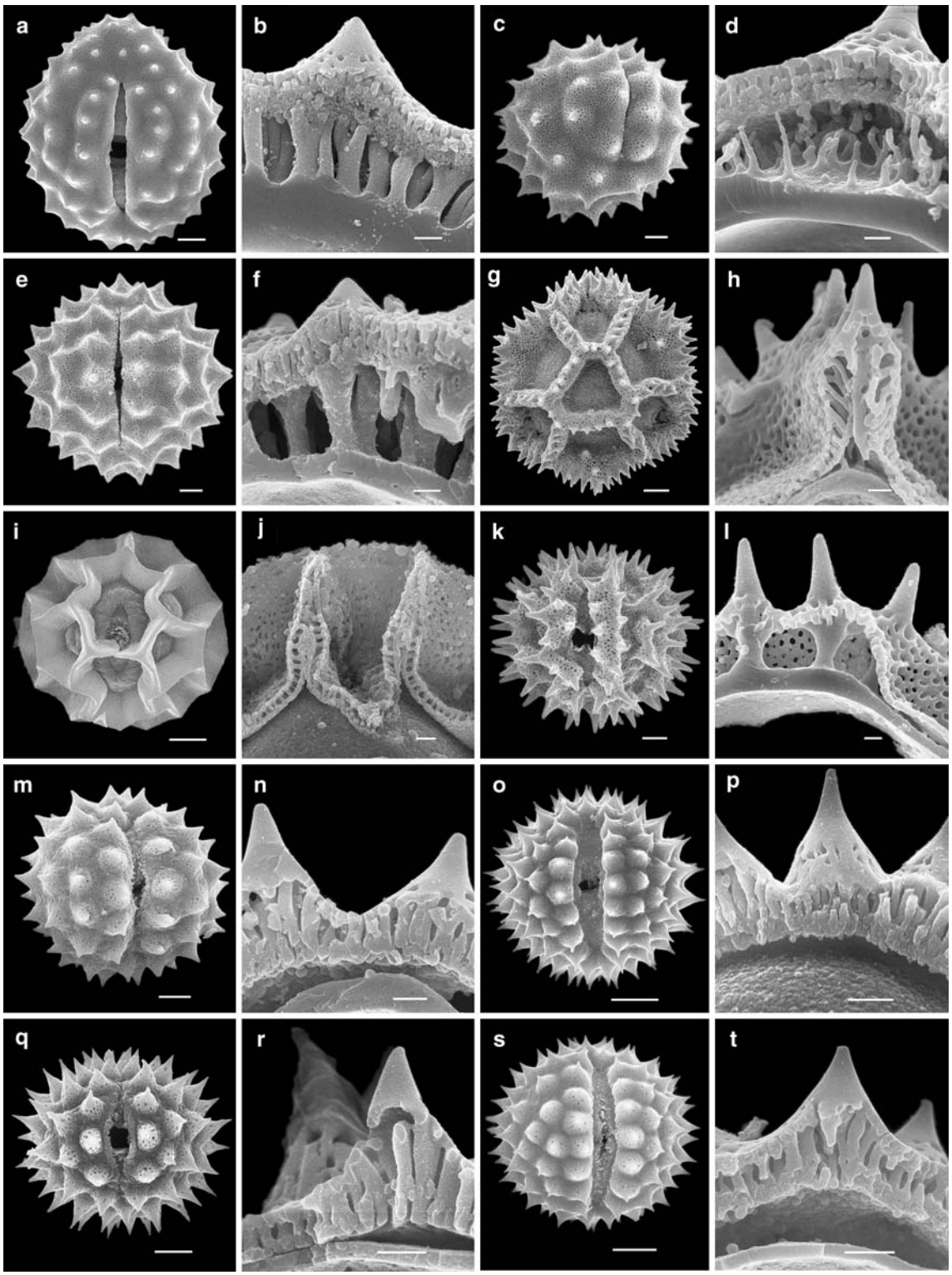


Fig. 1 Pollen structural diversity in Compositae (SEM micrographs). **a** *Dicoma sessiliflora* (Dicomeae/Mutisieae). **b** *Dicoma zeyheri* (Dicomeae/Mutisieae). **c**, **d** *Achyrothalamus marginatus* (Dicomeae/Mutisieae). **e** *Notobasis syriaca* (Cardueae). **f** *Arctium minus* (Cardueae). **g**, **h** *Scorzonera hispanica* (Lactuceae). **i** *Hirpicium bechuarense* (Arctoteae). **j** *Gazania rigens* (Arctoteae). **k** *Vernonia marginata* (Vernonieae). **l** *Vernonia arkansana* (Vernonieae). **m**, **n** *Cacalia plantaginea* (Senecioneae). **o**, **p** *Townsendia annua* (Astereae). **q**, **r** *Dittrichia graveolens* (Inuleae). **s** *Brickellia eupatorioides* var. *corymbulosa* (Eupatorieae). **t** *Eupatorium incarnatum* (Eupatorieae). *Scale bars* 5 μm (whole grains), 1 μm (fractured grains). Voucher details are shown in Table 1

and the exine is distinctly caveate (Fig. 2n, o, q–s). The Senecioid type differs from the Helianthoid type in lacking internal foramina (Fig. 2p, t). These three basic exine types have since been elaborated with a number of additional types (e.g. in tribe Cichorieae; Blackmore 1981, 1982; Nazarova 1997).

Wagenitz (1976) pointed out that, despite the palynological diversity in Compositae, fundamental characteristics shared between pollen grains support the continued recognition of a single family rather than division into two or more families. Within Compositae he described three evolutionary trends in pollen morphology: reduction of spines, reduction and loss of inner columellae (ultimately resulting in caveate pollen), and formation of surface ridges (lophae). Until recently, however, many researchers considered palynological characters to be highly homoplastic at sub-familial level and therefore of limited utility in phylogeny reconstruction or defining groups, or even obstructive to the correct delimitation of taxa (Turner 1977).

The publication of a supertree summarising the phylogeny of the Compositae (Funk et al. 2005) stimulated a new phase in the synthesis of knowledge concerning the family. In this context, Blackmore et al. (2009) recently reviewed palynological diversity, confirming that, when optimised on the supertree, pollen morphological characters can provide synapomorphies for almost every internal branch of the Compositae phylogeny. A striking conclusion of this study was that, although incongruent with classifications of the 1970s (Skvarla et al. 1977), the exine types proposed by Skvarla and co-workers are, in fact, highly congruent with today's supertree. Similarly, the evolutionary trends recognised by Wagenitz (1976) are strongly supported by patterns of pollen morphology optimised on the supertree, vindicating the importance of palynological evidence in studying the phylogeny and systematics of Compositae (Blackmore et al. 2009).

These developments prompted us to focus, in this paper, on the question of how the enormously diverse exine types in Compositae might be generated during the course of development. Given the wealth of information now available concerning the development of pollen walls (for recent reviews see McCormick 2004; Ma 2005; Blackmore et al. 2007), this topic can now be explored from both evolutionary and developmental perspectives. Information on pollen development is particularly extensive in Compositae because of the wide range of genera that have been studied, including *Artemisia* (Rowley and Dahl 1977; Rowley et al. 1981, 1999a), *Catananche* (Barnes and Blackmore 1988; Blackmore and Barnes 1988), *Cichorium* (Varotto et al. 1996); *Cosmos* (Dickinson and Potter 1976; Blackmore and Barnes 1985), *Dahlia* (Wodehouse 1930, 1931), *Eupatorium* (Skvarla et al. 2001), *Farfugium* (Takahashi 1989), *Gerbera* (Southworth 1983), *Haplopappus* (Wodehouse 1930), *Helianthus* (Horner and Pearson 1978), *Leontodon* (El-Ghazaly 1982); *Tagetes* (Heslop-Harrison 1969) and *Tragopogon* (Blackmore and Barnes 1987).

Inspired by the work of Thomson (1917), Wodehouse (1930, 1931, 1935), was perhaps the first to emphasise the importance to pollen development of pattern-formation processes resulting from physical and chemical interactions. This was a profound insight. As Blackmore et al. (2007) have emphasised, physical pattern formation (self-assembly) is involved at every level of the establishment and determination of pollen morphology and ultrastructure. As Wodehouse (1935) suggested, Heslop-Harrison (1968, 1971) and Dover (1972) subsequently confirmed that the organisational symmetry of pollen apertures is first determined during meiosis. Their number and position depends on interactions between the meiotic spindle and the mode and timing of cytokinesis, with the apertures being formed at the last points of cytoplasmic contact between tetrad members (at least in dicots; Blackmore and Crane 1988, 1998). Pollen surface morphology (sculpture) is superimposed upon this fundamental symmetry of aperture configuration and also reflects physical properties and interactions. In the "tensegrity model" proposed by Southworth and Jernstedt (1995), the initial patterning of the primexine is generated by the physical properties of the callose special cell wall (SCW) and the primexine matrix interacting with conditions of osmotic pressure and cytoskeletal tension within the microspore. Finally, self-assembly also plays an important role in determining the ultrastructure of the exine itself. This has been recognised in many studies of pollen development (e.g. Sheldon and Dickinson 1983; Dickinson and Sheldon 1986) but has only relatively recently been interpreted in terms of colloidal biology (Hemsley et al. 1992, 2003; Collinson et al. 1993; Scott 1994; Gabarayeva and Hemsley 2006; Hemsley and Gabarayeva 2007).

The processes by which the primexine progressively differentiates and the structural elements of the exine become recognisable were first elucidated using conventional electron microscopy (reviewed in Blackmore et al. 2007). Essentially similar sequences of events take place in all seed plants, and a particularly elegant account has been provided for *Brassica* by Fitzgerald and Knox (1995). Scanning electron microscopy of freeze-fractured anthers can provide a different perspective on the same sequence of events (Blackmore and Barnes 1985, 1987, 1988; Barnes and Blackmore 1988). Although primexine differentiation has been observed by these different techniques and described many times, it remained difficult to interpret until the recent development of models of self-assembly based on the behaviour of colloids (Hemsley et al. 2003;

Gabarayeva and Hemsley 2006; Hemsley and Gabarayeva 2007).

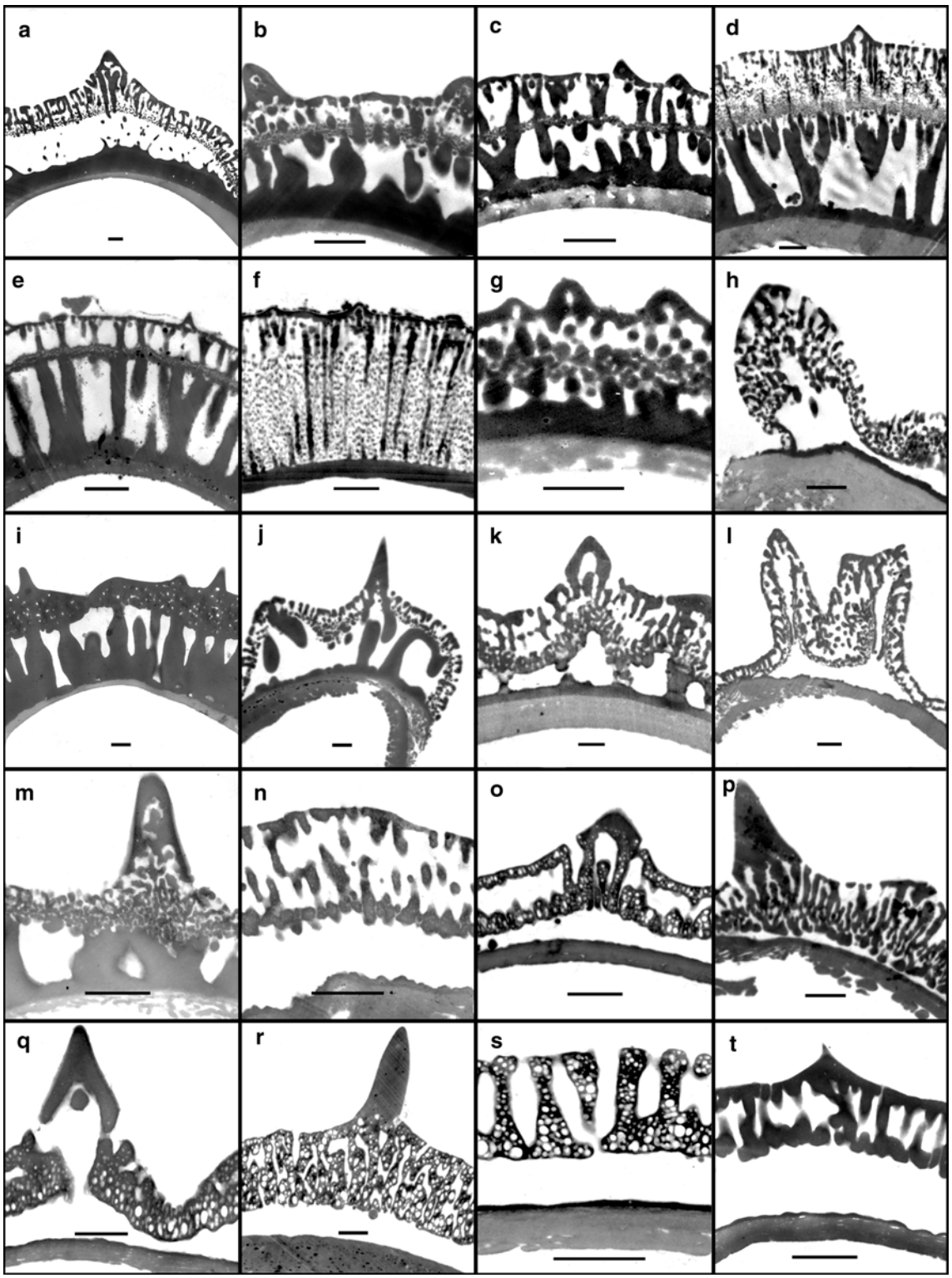
Application of colloidal self-assembly models enables several earlier competing interpretations of exine development and sub-structural organisation to be reconciled. For example, there has been a longstanding debate about whether the same processes are involved in the deposition of a tectate-columellate ectexine and in the endexine, which develops upon distinctive tripartite lamellae (Rowley and Southworth 1967), also known as white line centred lamellae. Blackmore (1990) pointed out that, in phylogenetic terms, white line centred lamellae represent the most plesiomorphic mode of exine deposition in land plants, with the derivation of an ectexine from the primexine having evolved as an innovation unique to seed plants. This perspective, whilst consistent with the pattern of land plant evolution, emphasises the differences between endexine and ectexine. In contrast, Dickinson (1976) recognised similarities between the mode of deposition of ectexine and endexine, reporting the presence of white line centred lamellae in differentiating elements of the ectexine. This observation can now readily be understood in terms of colloidal self-assembly. In one of the earliest exine self-assembly models published, Scott (1994) showed that the correspondence in size of tripartite lamellae and unit membranes reflects their parallel origins, which in both cases involve lamellar micelles (or neat micelles; Gabarayeva and Hemsley 2006) where the central white line corresponds to hydrophobic molecular tails. The diversity of micelles described by Gabarayeva and Hemsley (2006) may account for many, if not all, types of exine element. It also provides a satisfying explanation of how changes in phase that reflect the balance of lipids and water can reverse the organisation of micelles, accounting for the dramatic changes in electron density often seen in developing exines observed under TEM. The glycocalyx exhibits the properties of a lyotropic liquid crystal in which the relative concentrations of water and lipids cause significant changes to the structures that form by self-assembly of amphiphilic molecules that have immiscible hydrophilic and hydrophobic components. As the concentration of amphiphilic molecules increases within the aqueous medium of the glycocalyx, their arrangement moves from random in solution to ordered in micelles with the hydrophobic tails of the molecules facing inwards. The spherical micelles formed in this way initially remain discrete but, as the concentration of amphiphilic molecules increases past the "critical micelle concentration", cylindrical micelles are formed, as a layer in a more or less hexagonal arrangement (Gabarayeva and Hemsley 2006). Further increases in concentration can cause the micelles to adopt different arrangements such as lamellar or more complex, bicontinuous double-labyrinth micelles.

Fig. 2 Pollen structural diversity in Compositae (TEM micrographs, ▶ representing pollen types as defined in Skvarla et al. 1977). **a** *Achyrothalamus marginatus* (Dicomeae/Mutisieae), Anthemoid type. **b** *Adenocaulon bicolor* (Mutisieae), Anthemoid type. **c** *Perezia wrightii* (Mutisieae), Anthemoid type. **d** *Onoseris odorata* (Mutisieae), Anthemoid type. **e** *Trixis angustifolia* (Mutisieae), Anthemoid type. **f** *Doniophyton patagonicum* (Mutisieae), modified Anthemoid type. **g** *Tarchonanthus camphoratus* (Tarchonantheae), Anthemoid type. **h** *Chondrilla juncea* (Lactuceae), pollen type not described. **i** *Elephantopus nudatus* (Vernonieae), Anthemoid type. **j** *Cacosmia rugosa* var. *arachnoidea* (Liabeae), modified Anthemoid ("Liabioid") type. **k** *Carduncellus mitissimus* (Cardueae), Anthemoid type. **l** *Cullumia setosa* (Arctotideae), pollen type not described. **m** *Soliva stolonifera* (Anthemideae), Anthemoid type. **n** *Dimorphotheca pluvialis* (Calendulae), Helianthoid type (internal foramina barely visible). **o** *Carphephorus bellidifolius* (Eupatorieae), Helianthoid type. **p** *Tussilago farfara* (Senecioneae), Senecioid type. **q** *Chaenactis glabriuscula* var. *lanosa* (Helenieae), Helianthoid type. **r** *Fitchia speciosa* (Heliantheae), Helianthoid type. **s** *Helianthus giganteus* (Heliantheae), Helianthoid type. **t** *Ambrosia deltoidea* (Heliantheae/Ambrosiinae), Senecioid type. *Scale bars* 1 μm. Voucher details are shown in Table 1

It is clear that Heslop-Harrison (1972) was right to caution against assuming that genetic control extends to the finest details of pollen morphology. It now seems that epigenetic processes of pattern formation and self-assembly can account for many of the details of pollen symmetry, structure, and sculpture that have been employed as fixed characters for identification, classification, and phylogenetic study (Blackmore et al. 2007). Furthermore, through the insights from colloid science that have informed the theories of Hemsley and Gabarayeva (2007) and the tensegrity model of Southworth and Jernstedt (1995) it is becoming clear how physical forces can generate the complex microscopic patterns that Heslop-Harrison (1972) called "morphogenesis in miniature". In this paper we present a model for the origins of the structural diversity and complexity of pollen walls in Compositae building upon these recent insights and supported by observational evidence from developmental and mutant studies and the experimental dissolution of the exine.

Exine development in Compositae

First we consider the inferences that can be drawn from recent developmental studies. We follow the twelve stages of pollen development (Fig. 3) developed by Owen and Makaroff (1995) based on studies of *Arabidopsis* for the purposes of comparison between taxa or genotypes. The patterning and organisation of the pollen wall begins as early as during meiosis, with most of the characteristic structural complexity of the exine being determined in Stage 5 (the tetrad stage). Because the tetrad stage is of critical importance to the deposition and patterning of the



early exine, it is here divided into three sub-stages: Early, Mid, and Late Stage 5. From Stage 6, the early free microspore stage, until maturity (Stage 12), pollen wall development continues through the elaboration of the ectexine and the deposition of endexine and intine. The key stages in pollen development for wall deposition and differentiation are summarised in Fig. 4 and described in detail below, beginning at Owen and Makaroff's (1995) Stage 4 after meiosis, when patterning is initiated. Figure 5 shows, in diagrammatic form, the role of micelles in the

Table 1 Collections examined

Species	Location	Collection	Herbarium	Figures
Achryothalamus marginatus O.Hoffm.	Tanzania	Faulkner 3980	K	1d, 2a
Adenocaulon bicolour Hook.	Michigan, USA	Hiltunen 438	MSC	2b
Ambrosia deltoidea (Torr.) Payne	Illinois, USA	Payne 4978	ILLS	2t
Artemisia vulgaris L.	Stockholm, Sweden	No voucher	–	7a–f
Arctium minus Hill (Bernh.)	Delaware County, Oklahoma, USA	Olney 74	OKL	1f
Brickellia eupatorioides L. (Shinners) *var. corymbulosa* (Torr. & A.Gray) Shinners	Oklahoma, USA	Johnson, Proctor & Vezey GRU 0059	OKL	1s
Cacalia plantaginea (Raf.) Shinners	Ottawa County, Oklahoma, USA	Wallis 7257	OKL	1m, n
Cacosmia rugosa H.B. & K. var. *arachnoidea* Hieron.	Ecuador	Wiggins 10810	TEX	2j
Carduncellus mitissimus DC.	France	Chavier, J. s.n.	TEX	2k
Carphephorus bellidifolius Torr. & A.Gray	North Caroline, USA	Radford 28891	TEX	2o
Chaenactis glabriuscula DC. var. *lanosa* H.M.Hall	California, USA	Wolf 6966	OKL	2q
Chondrilla juncea L.	Texas, USA	University of Texas accession number 193928	TEX	2h
Cullumia setosa (L.) R.Br.	South Africa	Ryder s.n.	K	2l
Dicoma sessiliflora Harv.	Malawi	Chikuni, A. & Nachamba, W. 190	MO	1a
Dicoma zeyheri Sond.	South Africa	Burrell, M. 2673	GH	1b
Dimorphotheca pluvialis (L.) Moench	South Africa	Hutchinson 550	K	2n
Dittrichia graveolens (L.) Greuter	Greece	Greuter, W. 10654	OKL	1q, r
Doniophyton patagonicum (Phil.) Cabrera	Argentina	Correra & Nicora 3712	CONC	2f
Elephantopus nudatus A.Gray	Oklahoma, USA	Coryell 558	OKL	2i
Eupatorium incarnatum Walter	Oklahoma, USA	Crutchfield, J. R. 2285	OKL	1t
Fitchia speciosa Cheeseman	Hawaii, USA	Carlquist 1684	RSA	2r
Gazania rigens (L.) Gaertn.	University of Oklahoma greenhouse, Oklahoma, USA	Cult.	OKL	1j
Helianthus giganteus L.	Oklahoma, USA	Bebb 6079a	OKL	2s
Hirpicium bechuanense (S.Moore) Roessler	Southern Rhodesia	Drummond, C. R. 5735	K	1i
Notobasis syriaca (L.) Cass.	Italy	Brummit, R. K. 4576	K	1e
Onoseris odorata (D.Don) Hook. & Arn.	Peru	Ferreyra 6353	OKL	2d
Perezia wrightii A.Gray	Texas, USA	Whitson s.n.	OKL	2c
Scorzonera hispanica L.	Chelsea Physic Garden, London, UK	Cult.	BM	1g, h; 6a–k; 8a–d
Scorzonera humilis L.	Dorset, UK	Blackmore s.n.	BM	7k, l
Soliva stolonifera R.Br. ex Sweet	Alabama, USA	Harper 3351	MO	2m
Tarchonanthus camphoratus L.	Kenya	Maas Geesteranus, R. A. 6186	US	2g
Townsendia annua Beaman	New Mexico, USA	Osterhout, G. E. 6954	OKL	1o, p
Trixis angustifolia DC.	Mexico	Rinehart 7019	OKL	2e
Tussilago farfara L.	Czechoslovakia	Deyl 97	TEX	2p
Vernonia arkansana DC.	Oklahoma, USA	McCarty GRU 0757	OKL	1l
Vernonia marginata (Torr.) Raf.	Oklahoma, USA	Taylor & Taylor 4636	OKL	1k

self-assembly of ectexine elements. Key features in pollen wall deposition are also illustrated using the examples (from Compositae) of *Scorzonera* spp. (Figs. 6, 8) and *Artemisia vulgaris* (Fig. 7), by TEM and SEM.

Stage 4 (meiosis complete)

From Stage 4 the SCW is completed by synthesis of callose from within the microsporocyte and, later, its four daughter

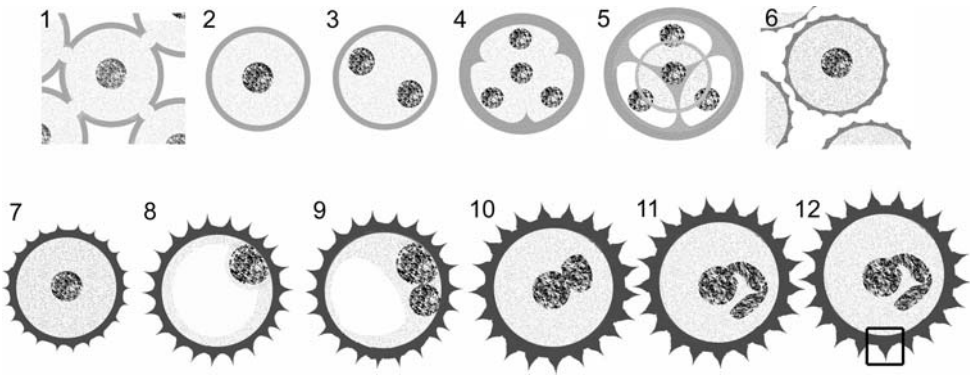


Fig. 3 The twelve stages of pollen development (after Owen and Makaroff 1995), redrawn from Blackmore et al. (2007). **1** Premeiosis I: microsporocytes connected by cytomictic channels. **2** Premeiosis II: microsporocyte surrounded by callose SCW. **3** Meiosis I: reduction division in progress in a microsporocyte. **4** Meiosis complete: before cytokinesis. **5** Tetrad stage (divided in this paper into Early, Mid and Late): callose SCWs present around microspores. **6** Free microspore I: microspores surrounded by differentiating exine. **7** Free microspore II: further differentiation of exine. **8** Ring-vacuolate microspore, with large vacuole causing characteristic "signet ring" appearance. **9** Bicellular pollen I: asymmetric mitosis gives rise to a vegetative cell surrounding a peripheral generative cell. **10** Bicellular pollen II: generative cell central. **11** Second mitotic division: formation of male germ unit. **12** Mature pollen, with storage products accumulated in cytoplasm and surface tryphine. The box shows the area detailed in Fig. 4

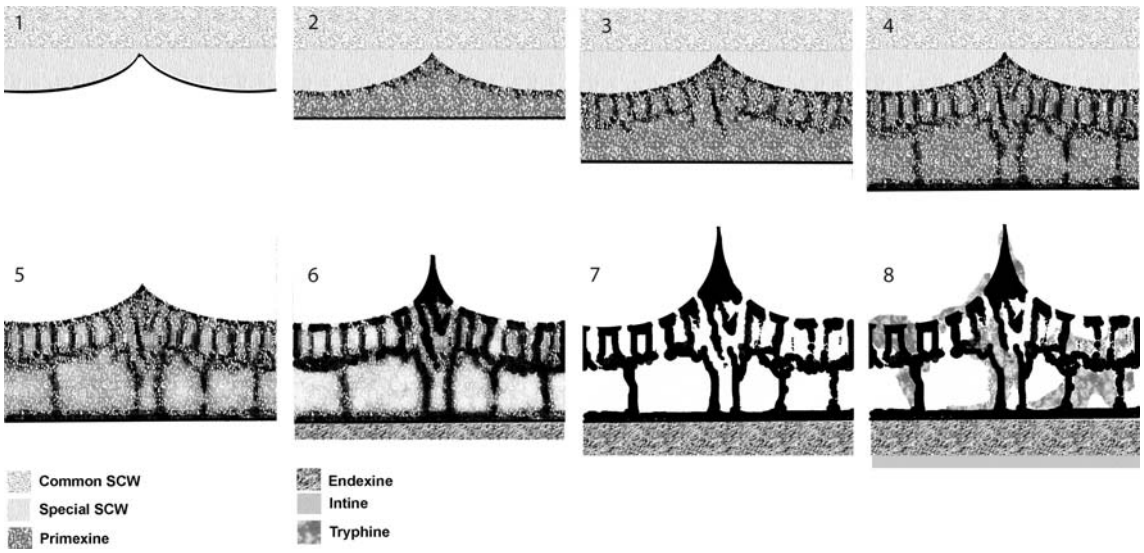


Fig. 4 Diagrammatic summary of the main stages of pollen wall differentiation in Compositae, based on *Scorzonera hispanica*. **1** Two-layered callose SCW comprising a common SCW initiated during Stage 3 of pollen development and an individual SCW deposited late in Stage 4. **2** Initial deposition of primexine during Early Stage 5. **3** Further deposition and differentiation of primexine during Mid Stage 5. **4** Completion of primexine deposition during Late Stage 5. **5–8** Free microspore period. **5** Differentiation of primexine following the dissolution of the SCWs and release from tetrads during Stage 6. **6** Further differentiation of exine and commencement of endexine deposition during Stages 7 and 8. **7** Completion of the endexine during Stages 8–10. **8** Mature pollen, at Stage 12, with tryphine and intine

cells (Blackmore et al. 2007; Figs. 3[5], 4[1]). As Longly and Waterkeyn (1979) illustrated in their classic paper on cytokinesis in anthers, the callose SCW comprises two structures, a common SCW deposited around the microsporocyte and an individual SCW around each microspore of the tetrad (Fig. 6b–c). The common SCW often varies in thickness but always encloses a spherical space within which meiosis takes place. It is generally assumed that the differential deposition of callose is controlled by the plasma membrane through which callose or its precursors are secreted. How this control operates is unknown, but Paxson-Sowders et al. (1997) have used mutant analysis in

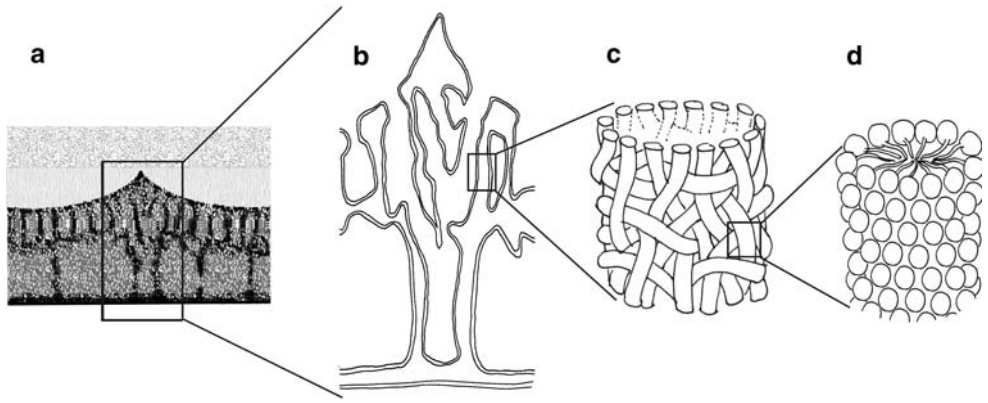


Fig. 5 Diagram showing sub-structural organisation formed by self-assembly at different scales in the differentiating primexine. **a** Primexine during late tetrad stage (Stage 5) corresponding to Fig. 4[4]. **b** Enlargement showing outline of the boundary layer formed within the differentiating primexine. **c** Enlargement of one section of a differentiating columella showing meshwork organisation of individual elements that make up the hollow, cylindrical structure of the boundary layer. **d** Enlargement of one element of the meshwork boundary layer, corresponding to a single tuft, shown as a cylindrical micelle

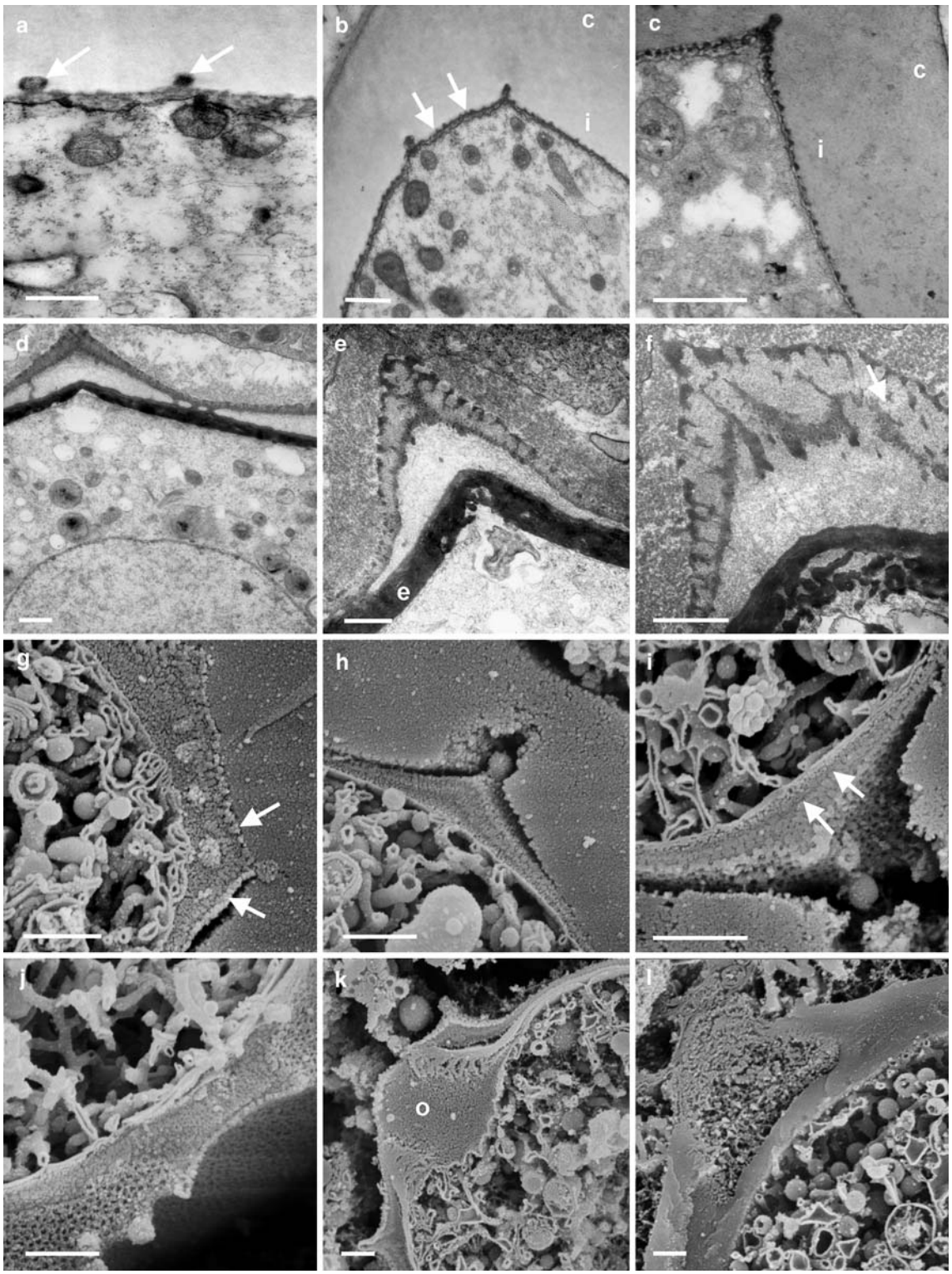
Arabidopsis to confirm the importance of the invaginations of the plasma membrane in determination of normal exine patterning. The individual SCWs are of more uniform thickness than the common SCW, except in those Compositae with echinolophate pollen (Blackmore and Barnes 1987), where areas of thicker callose deposition on the inner surface of the individual SCWs correspond to the position of the lacunae between the lophae of the mature pollen (Fig. 6g, h).

Early Stage 5 (early tetrad stage)

At the beginning of the tetrad stage (Fig. 4[2]), the microspores switch from synthesising callose to a primexine matrix (also known as the glycocalyx, cell surface coating, or extracellular matrix (ECM); Fig. 6a–c), a microfibrillar material composed largely of glycoproteins (Rowley 1971, 1973; Pettitt and Jermy 1974; Rowley and Dahl 1977; Pettitt 1979), which functions as an elaborate matrix in which the patterned accumulation of sporopollenin precursors and their subsequent polymerisation takes place (Heslop-Harrison 1968; Rowley and Skvarla 1975; Rowley and Dahl 1977; Blackmore et al. 2007).

The positions of the spines of Compositae pollen are established at the very beginning of Stage 5, when the deposition of the primexine matrix has begun (Fig. 6a, b). In echinate pollen grains, for example those of *Tagetes*, they are regularly arranged over the microspore surface in a repeating hexagonal pattern (Heslop-Harrison 1979; Takahashi 1989) whereas in echinolophate pollen grains they are arranged along the crests of the future lophae, bounding the polyhedral lacunae (Barnes and Blackmore 1986; Blackmore and Barnes 1987). In both cases we consider the tensegrity model of Southworth and Jernstedt (1995) to be the likely means by which a pattern of points (corresponding to the positions of spines) is imprinted on the plasma membrane and reflected in terms of differential callose deposition. The absence of spines from apertural regions (both the simple ectocolpi of echinate pollen grains and the lacunate ectocolpi of echinolophate pollen) may be explained by the presence of apertural shields of endoplasmic reticulum which prevent or greatly restrict primexine deposition (Heslop-Harrison 1963, 1968) at the sites of the future ectoapertures.

Fig. 6 Exine deposition in *Scorzonera* (TEM and SEM micrographs). ▶ **a** *Scorzonera hispanica* TEM showing very young tetrad microspore with early glycocalyx layer and spherical progenitors of spines (*arrowed*). **b** *Scorzonera hispanica* TEM showing young tetrad stage; small spherical units (*arrowed*) can be seen in the glycocalyx layer between the larger spine progenitors; *c common SCW*, *i individual SCW*. **c** *Scorzonera hispanica* TEM showing mid tetrad stage, with spherical units in the glycocalyx increasing in prominence and marking the sites of future columellae; *c* common SCW, *i* individual SCW. **d** *Scorzonera hispanica* TEM showing differentiating outer ectexine, cavea traversed by slender columellae, foot layer and endexine. **e** *Scorzonera hispanica* TEM showing further differentiation of primexine in the region of a spine; endexine also present. **f** *Scorzonera hispanica* TEM showing well-developed spine and endexine dissipating into lamellae; callose still present; *arrows* indicate continuous system of interconnected empty spaces within exine. **g** *Scorzonera hispanica* SEM showing SCW, and differentiating primexine, with tectum already distinct (*arrowed*). **h** *Scorzonera hispanica* SEM showing primexine differentiation in the late tetrad stage with callose still present but beginning to disperse from a developing ridge. **i** *Scorzonera hispanica* SEM showing callose dispersing to reveal a microreticulate tectum. Within the primexine an internal tectum can be distinguished (*arrowed*). **j** *Scorzonera hispanica* SEM young microspore after dissolution of the callose, with primexine differentiating beneath the microreticulate tectum. **k** *Scorzonera hispanica* SEM, section through developing aperture showing large granular oncus (*o*) interbedded with endexine lamellae. **l** *Scorzonera humilis* SEM, section through developing aperture showing thickness of mature endexine. *Scale bars* 1 μm. Voucher details are shown in Table 1



There is ample evidence that the glycocalyx is not of uniform composition throughout its period of deposition. For example, Gabarayeva and Grigorjeva (2002, 2003) have reported sequentially different phases of glycocalyx deposition in *Illicium* (Illiciaceae) and *Stangeria* (Stangeriaceae).

The centripetal deposition, first of callose and then of the primexine matrix, which provides a template for the deposition of the sporopollenin primexine, establishes a temporal sequence that persists throughout pollen wall development. As a consequence the structure of the mature pollen grain can itself be considered to represent a temporal

progression with the oldest material at the outer surface (the tectum), and the infratectum, foot layer, endexine I, and endexine II being progressively younger (Fig. 4). One obvious exception to interpreting the exine in terms of a programmed temporal sequence arises because the tapetum also contributes sporopollenin to the pollen wall, so that supratectal features, in particular, may be deposited out of sequence. For instance in *Artemisia* (Fig. 7), sporopollenin deposition occurs during a second period late in the development of the pollen grain, and more typically in early development (Rowley and Dahl 1977; Fig. 7d).

In all flowering plants that have been studied, dark staining globules of material are deposited outside the plasma membrane during this stage (Fig. 6a, b). These have been interpreted by Gabarayeva and Hemsley (2006) as spherical micelles composed of hydrophobic liquid hydrocarbon nuclei with hydrophilic groups on the outer surface (Fig. 5d). These represent the first stages in the organisation of sporopollenin-receptive sites within the glycocalyx. Progressively, through the early tetrad stage, these spherical micelles change their disposition, forming an interconnected network (Fig. 5c) that has been widely observed in early primexines (reviewed by Rowley 1990). The elements that make up this network may now be interpreted as cylindrical micelles (Fig. 5d; c.f. Gabarayeva and Hemsley 2006: Fig. 1) which individually correspond to the exine substructures that Rowley and co-workers called "tufts" (Rowley et al. 1981, 1999c; Fig. 5d). Blackmore (1990) proposed that such a network, formed during the early differentiation of the primexine, establishes a "boundary layer" defining the initial form of the ectexine elements (Fig. 5b). This boundary layer also shows a centripetal pattern of differentiation, so that the definition of the structural elements comprising the microreticulate tectum is established first. As primexine differentiation progresses, two things happen: the boundary layer extends centripetally, forming the structural outlines of columellae, granules, or other ectexine elements (Fig. 4[4, 5]) and soon afterwards sporopollenin precursors begin to accumulate (Figs. 6c, 7c).

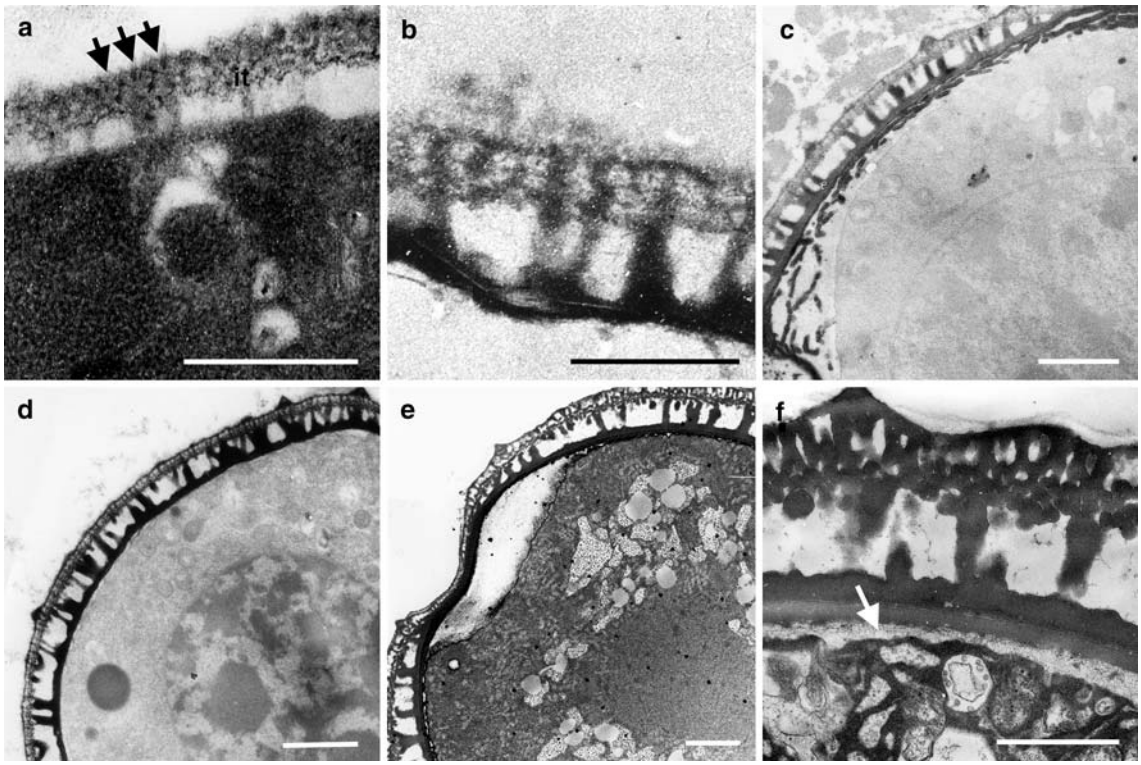


Fig. 7 Later stages of exine deposition in *Artemisia vulgaris* (TEM micrographs). **a** Differentiating primexine with radially orientated tufts (*arrowed*) in the outer ectexine, internal tectum (*it*) and cavea traversed by a few thicker columellae. **b** Slightly more mature primexine with partially differentiated columellae; endexine deposition on tripartite lamellae has commenced; columellae attached to the endexine and foot layer extends through the complex inner tectum; outer tectum and rudiments of spinules are formed by rod-like units of the exine. **c** Free microspore prior to accumulation of sporopollenin into the framework established by the boundary layer. **d** Late microspore after additional sporopollenin incorporation into ectexine structures and spines. **e** Maturing pollen grain. **f** Mature pollen grain, with exine showing great increase in sporopollenin deposition late in development throughout, including spinules, outer and inner tectum, columellae, foot layer and endexine. *Arrows* indicate pecto-cellulosic intine. *Scale bars* 1 μm. Voucher details are shown in Table 1

Mid Stage 5 (mid tetrad stage)

During the mid tetrad stage (Fig. 4[3]) the glycocalyx continues to increase in thickness, and within it the process of micelle formation continues, extending the meshwork boundary layer centripetally. The organisation of the boundary layer, with sporopollenin-receptive sites contained in micelles, progressively defines the columellae, granules, or other structures that correspond to the outer ectexine of the mature pollen grain (Fig. 5). As Figs. 1 and 2 show, this part of the exine has both a high degree of complexity and a wide variety of different forms in Compositae. In exines with a simpler, tectate-columellate organisation, the process of glycocalyx deposition and differentiation is much simpler and briefer (e.g. *Arabidopsis*; Owen and Makaroff 1995; Blackmore et al. 2007). We suggest that the diversity of forms of boundary layers, and subsequently of mature exines, in Compositae reflect very subtle taxon-specific variations in the composition and properties of the glycocalyx.

The discrete nature of the two domains of the exine—ectexine and endexine—can be interpreted as a phase transition in a lyotropic system to another type of micelle. The latter—neat (lamellar) micelles—comprise parallel bilayers of surfactant molecules arranged hydrophobic tail to hydrophobic tail, with aqueous layers between, giving the appearance of tripartite lamellae when seen under TEM (Gabarayeva and Hemsley 2006). These structures appear abruptly in the periplasmic spaces, as a result of an increase in the concentration of surface-active glycoproteins in the medium at this stage in development. The sudden nature of the transition is connected with the non-linear nature of self-assembly, whereby small changes in conditions may have no or a great effect (Hemsley 1998). It has been suggested that increasing concentrations of cations including calcium ions cause the surface aggregation of glycoprotein molecules into micelles (Florence 1977; Paxson-Sowders et al. 2001).

Minor changes in ionic concentration within the glycocalyx, perhaps coupled with the effects of tensegrity, can produce boundary layer defined ectexine structures ranging from granules to much branched columellae and internal tecta (Fig. 2). The major types of ectexine organisation seen in Compositae are: uniformly granular, columellate and/or spongy from the tectum to the foot layer with no distinct internal tectum (Fig. 2f); having a single distinct internal tectum (Fig. 2b, c, e, o, q, s, t); or having more than one internal tectum or a complex granular or multilayered outer ectexine (Fig. 2a, d, g, i–n, p, r). Where one or more internal tecta are present each usually marks a distinct change in the scale of columellae present (Figs. 1b, 2b, d, i). Below the internal tectum, columellae may be entirely absent, leaving a cavea (Figs. 1n, p, r, t, 2n, q–t). A cavea is, in effect, an area devoid of glycocalyx subunits where, consequently, there is no framework or template for the accumulation of sporopollenin. Alternatively the columellae may abruptly become much larger in diameter as the elaboration of the boundary layer beneath the internal tectum continues to define the fewer, more widely spaced columellae of the infratectum (Fig. 2i).

Although Rowley and Southworth (1967) discussed sporopollenin receptors and they have been referenced many times since (see, for example, Dickinson and Sheldon 1986), their precise nature still remains unknown. The concept of specific sporopollenin acceptor particles (SAPs) was first used in relation to palynology in *Borago* (Boraginaceae), more than 20 years later (Rowley et al. 1999b). It is clear that the glycocalyx facilitates the initial deposition of sporopollenin monomers, and that the uneven distribution of polymer initiation sites (in boundary layers) within the glycocalyx gives rise to the structure seen in the developing exine (Gabarayeva and Hemsley 2006). This primary structure is, in essence, the primexine, a preliminary draft of the future exine, and the polymer initiation sites are SAPs. While the sporopollenin of mature exines can be degraded (e.g. by oxidation with potassium permanganate; Fig. 8), the low concentrations of sporopollenin associated with SAPs are resistant to such treatments (Southworth 1974, 1986; Rowley and Dahl 1977; Rowley and Prijianto 1977; Blackmore and Claugher 1987; Blackmore 1990; Gabarayeva et al. 2003). Thus the primexine, in contrast with the bulk of sporopollenin accumulated during the free microspore stage, is very stable. This suggests a compositional difference which may indicate receptor-dependent and receptor-independent sporopollenin types, perhaps relating to different sporopollenin monomer sources (Rowley and Claugher 1991; Rowley and Skvarla 1993). Histochemical studies have shown that SAPs contain proteins and are probably enzyme catalysts for sporopollenin monomer polymerisation (Rowley et al. 1999b). It has also been shown that SAPs occur distributed alongside the walls of cylindrical tuft-micelles in the glycocalyx development of *Encephalartos* (Cycadaceae), bringing about sporopollenin accumulation and the appearance of cylindrical alveolae in the exine pattern (Gabarayeva and Grigorjeva 2004). In this case, an actual boundary layer formed of SAPs has been observed surrounding each cylindrical glycocalyx unit, producing the alveolate exine.

During the mid tetrad stage and continuing into the late tetrad stage the accumulation of sporopollenin in the developing ectexine is often associated with a reversal in the staining properties of the primexine and glycocalyx units, because of the micelle inversion in the supporting liquid (Gabarayeva and Hemsley 2006).

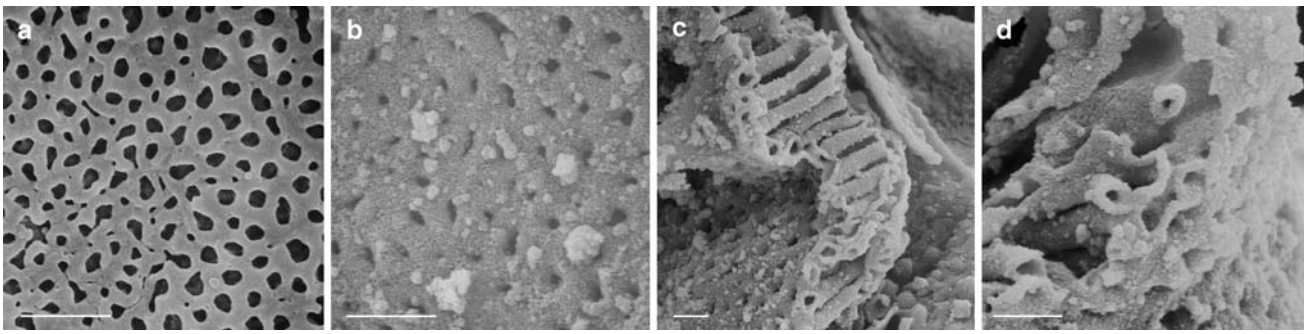


Fig. 8 Exine deposition and dissolution in *Scorzonera hispanica* undergoing treatment with potassium permanganate (SEM micrographs). **a** Surface of grain ×20, untreated. **b** Surface of grain ×20, treated with potassium permanganate showing removal of superficial sporopollenin to re-expose the meshwork boundary layer. **c** Detail of columellae ×10, treated with potassium permanganate showing meshwork boundary layer and columellae reduced to hollow cylinders. **d** Detail of columellae ×20, treated with potassium permanganate showing the continuous space within the ectexine that is enclosed by the boundary layer. *Scale bars* 5 μm (**a**), 1 μm (**b**–**d**). Voucher details are shown in Table 1

Late Stage 5 (late tetrad stage)

By the end of the tetrad stage (Fig. 4[5]) the definition of the structural elements of the ectexine is complete although they differ substantially from those in the fully mature exine (Figs. 6d–f, 7b). The spines, for example, are relatively low and appear peg-like rather than acute (Fig. 6j). In thin sections the ectexine elements such as columellae appear less solid and substantial than in mature pollen grains (Figs. 6f, 7e). These differences are readily explained by the relatively limited amount of sporopollenin accumulated and polymerised within the ectexine at this stage. The boundary layer defines a hollow and continuous system of interconnected empty spaces (arrow in Fig. 6f) that extend from the tectum, through the columellae and internal tectum, to the foot layer.

An abrupt change in the pattern of micelle formation marks the onset of endexine deposition on the highly characteristic white line centred or tripartite lamellae. Near the developing endoapertures these lamellae are widely separated and interspersed with material of the oncus (or zwischenkorper), which does not accumulate sporopollenin.

Stage 6 (early free microspore stage)

After the release of the microspores from the callose SCW at the end of the tetrad stage (Fig. 4[6]), sporopollenin precursors are incorporated into the developing ectexine from the surrounding tapetum (Dickinson and Heslop-Harrison 1968; Heslop-Harrison 1968; Dickinson and Potter 1976) and from within the microspores. This additional sporopollenin forms a smooth surface over the meshwork boundary layer, obscuring it. However, the deposition of additional sporopollenin from the tapetum is not of uniform thickness over the entire pollen grain. The tips of spines attract more sporopollenin precursors than the surface of the micro-reticulate tectum. This reflects the observation by Gabarayeva and Hemsley (2006) that sharp topology can change the way in which charged particles accumulate, causing the preferential accumulation. Interpreted in this way, even the extension of spines and the development of their acute apices involve a process of self-assembly.

It is also interesting to note that the accumulation of sporopollenin inside the continuous hollow space of the boundary layer can take two distinct forms in the Compositae. In some cases the space is completely filled so that the columellae, internal tecta or other infratectal structures become solid (Fig. 2t). In others, spaces remain where no sporopollenin is accumulated, so internal foramina are formed (Fig. 2s). It is likely that only slight differences in micelle formation account for these differences which result in very different mature pollen wall structures. In the case of internal foramina, it is possible that they are formed by addition of a water-based component to the lipid-based medium of the premature exine, forming a reversed water-in-oil emulsion, with the water droplets preventing sporopollenin accumulation. This suggestion accords with the observations of Rowley and Skvarla (2007) that exines remain fluid until very late in their development. The same conclusion (i.e. that exines have a liquid crystal structure) was also suggested by ontogenetic sporoderm studies in *Trevesia* (Araliaceae; Gabarayeva et al., submitted for publication).

Stages 7–12

From Stage 7 to maturity the differentiation of the ectexine continues through the addition of sporopollenin and the dispersion of material that is not receptive to sporopollenin (Fig. 6h, i), creating spaces within the ectexine (Fig. 4[7]).

These spaces are particularly large in pollen grains with a cavea. As the tapetum degenerates, it forms pollenkitt or tryphine which coats the outer surface and penetrates into the spaces within the pollen wall (Fig. 4[8]).

After the developing pollen grains become tricellular, the final, innermost layer of the pollen wall is formed (arrow in Fig. 7f). This is the pecto-cellulosic intine; its development marks another shift in synthetic activity mediated via the plasma membrane, this time resulting in a more typical plant cell wall.

Observations based upon exine dissolution

In addition to the developmental studies discussed above, observations from physical and chemical dissolution of the exine have long been used to investigate exine substructure (Rowley and Prijianto 1977; Rowley 1980, 1990; Rowley et al. 1981, 1999c; Blackmore and Claugher 1987). One of the simplest methods involves treating exines with potassium permanganate which has the effect of removing superficial sporopollenin and exposing the meshwork material of the boundary layer (see, for example, Blackmore and Claugher 1987, Fig. 20). Frequently, treatment with potassium permanganate removes sporopollenin from the centre of solid ectexine elements such as columellae (Fig. 8c, d; the *Scorzonera hispanica* examples here have previously been published in Blackmore and Claugher 1987). Hollow columellae have also been observed in the exine of *Lavatera arborea* (Malvaceae) after experimental oxidation (Gabarayeva et al. 2003). Blackmore and Claugher (1987) concluded that the effect of potassium permanganate is to remove all of the exine except for the boundary layer formed during the differentiation of the primexine. They noted that the endexine, which is removed completely during potassium permanganate treatment, lacks a boundary layer. Similar results were presented by Rowley (1990). Blackmore (1990) illustrated the meshwork boundary layer in both freeze-fractured developing primexines and in potassium permanganate-treated mature pollen of *Echinops sphaerocephala*. These examples provide evidence that the boundary layer recovered during exine dissolution represents re-exposure of a structure formed early in pollen ontogeny.

Discussion

The concept of the pollen wall representing a centripetal developmental sequence provides an interesting and informative model in which the deposition and differentiation of the pollen wall progress spatially and temporally from tectum to intine (Figs. 4, 5).

When interpreting the structure of mature exines as a temporal sequence of this kind, a number of key transitions can be found that result in different structures being formed (in the ectexine a sequence from tectum to outer columellae or ectexine granules, to internal tectum, to inner columellae and foot layer is observed). These key transitions correspond to specific points in the programme of patterned deposition of materials on the outer surface of the plasma membrane where there is a change that affects micelle formation. Micelle formation might change through the sequence of glycocalyx deposition and primexine differentiation in terms of ionic concentrations, the precise substances that are being synthesised, or a combination of such factors. One distinct switch occurs, for example, at the point when callose synthesis ends and glycocalyx synthesis begins. There is, therefore, scope for much complexity in the pattern-forming processes of self-assembly by micelle transitional mesophases such that abrupt transitions in form are generated. These can be regarded as "switches" in the sense that they produce a significant change in form but they may reflect only the slightest change in the conditions within which micelle formation takes place.

However, because the tapetum also contributes sporopollenin to the ectexine, from the free microspore stage onwards (and perhaps even during the late tetrad stage, because there is evidence that material can pass through the callose SCW) the structure of the mature ectexine cannot be read as a simple continuous sequence from the outside inwards. Instead it is necessary to recognise that this fundamental sequence may be overlain by later deposited sporopollenin. Nevertheless, the structure and sculpture of the pollen grain are mostly determined by the primary temporal sequence, with tapetally derived sporopollenin essentially strengthening or adding to the original pattern.

Considered in the light of this model it becomes possible to see how the vast diversity of exine structures in Compositae, of which Figs. 1 and 2 provide a glimpse, might be generated. Some patterns and motifs in ultrastructure are repeated (for example, Fig. 1a, f, p, t), either with or without internal foramina. The outer ectexine may variously be formed of granules (Fig. 2f), distinct columellae (Fig. 2e) or a combination of the two (Fig. 2d). Sometimes a distinct internal tectum is formed (Fig. 2e) and the space beneath it might be occupied by the void of a cavea or by columellae, generally much larger and more widely spaced than those of the outer ectexine. Both caveae and internal tecta, and internal foramina, are absent from the typically prolate, psilate, and microperforate pollen of basally branching lineages in Compositae (Blackmore et al. 2009) and can therefore be regarded as relatively derived features within the family. In derived groups such as Asteroideae, pollen tends to be oblate in shape, microreticulate and

echinate, usually with caveae and internal foramina. Thus, successive branches of the Compositae supertree show shifts towards an exine ultrastructure with few-branched columellae, caveae, internal tecta, and internal foramina, plus external changes in spine shape, size, and internal structure. The ontogenetic differences between those early branching Compositae that lack internal foramina and caveae, and the more derived taxa that possess them, are best thought of as subtle changes in the colloidal environment for micelle formation, rather than precise differences defined by specific genes. Note, however, that Jackson et al. (2000) have also shown that a single-gene mutant can disrupt the organisation of ectexine, resulting in pollen lacking the cavea and foot layer of normal pollen in *Haplopappus* (Compositae). It remains uncertain whether such mutations affect the formation of sporopollenin precursors, the colloidal conditions within the developing or glycocalyx, or some other aspect of microspore development. The examples of *Doniophyton* and *Onoseris* provide a particularly good demonstration of very different forms resulting from subtle changes to the developmental programme of self-assembly: the former (Fig. 2f) lacks cavea and an internal tectum so that the ectexine is uniform from tectum to foot layer, comprising slender columellae interspersed with granules; the latter (Fig. 2d) has an outer ectexine identical in appearance but an abrupt switch has generated an internal tectum, below which are large, widely spaced columellae. There can be little doubt that similar patterning processes underlie the entire ectexine in *Doniophyton* and the outer ectexine in *Onoseris*.

It is clear from very many different fields of biology that self-assembly is a widespread and fundamentally important phenomenon which can operate in a hierarchical manner at a variety of different scales. In one recent example, Capito et al. (2008) have shown how self-assembly of membranous sacs can occur at the boundary between two aqueous solutions, one containing a polymer of large molecules and the other small self-assembling molecules of opposite charge. The resulting sacs have a highly structured organisation comprising aligned bundles of nanofibres. Their experiments demonstrate self-assembly on several different spatial scales, resulting in the formation of a membrane which prevents diffusion between the two liquids followed by the self-assembly of microfibres. The orientation of these microfibres can exhibit abrupt transitions from a more or less radial or irregular organisation in those that are formed first to strongly aligned microfibres lying perpendicular to them. This abrupt switch in orientation of self-assembled structures shows some parallels to the transition from ectexine to endexine deposition, although it is unlikely that the two systems are directly comparable.

The insights provided by this model into the diversity of exine structure in the Compositae do not undermine the taxonomic utility of characters based upon differences in exine structure. Whilst it is possible that much of the diversity of form encountered in plants, especially on the microscopic scale, is the product of self-assembly, the conditions for this self-assembly are presumably heritable. In other words, both the synthesis of materials at the surface of microspore and tapetal cells and the balance of ionic concentrations within the colloidal environment of the glycocalyx and primexine are under direct genetic control whereas the complex interplay between them that generates particular structures is not. The DEX1 protein, for example, was considered by Paxson-Sowders et al. (2001) to have a role in the transport, attachment, or polymerisation of sporopollenin precursors and to influence Ca^{2+} ion concentrations within the primexine.

In presenting this model we suggest that a major remaining challenge for the future will be to understand how gene-controlled processes operating during flower, stamen, and pollen ontogeny establish the precise milieu within which self-assembly processes unfold.

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