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Bot. Rev. (2014) 80:107–134
DOI 10.1007/s12229-014-9135-1

Orbicules in Flowering Plants: A Phylogenetic Perspective on their Form and Function

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Published online: 22 May 2014

Abstract Next to pollen, stamens of flowering plants often produce microstructures, called orbicules, lining the locules. Although the existence of orbicules has been known since 1865, their function still remains enigmatic. This paper surveys orbicule distribution throughout angiosperms, including +1,500 entries. We show that orbicules are found all over of flowering plants with an evolutionary trend towards orbicule absence in more derived clades. Orbicules are common in the ANITA-grade and 85 % of the monocots studied produce orbicules, with Orchidaceae, Commelinales and Zingiberales as notable exceptions. Within eudicots, asterids are most densely sampled with 61 % orbicule presence. Asteraceae and the majority of Lamiaceae lack orbicules. For 17 angiosperm orders orbicule distribution data are lacking entirely. We demonstrate that the hypothesized correlation of orbicule presence with non-amoeboid tapetum types holds true. The presence of orbicules is therefore a convenient proxy for tapetum characterization. The potential of orbicules as an a-cellular model system for patterned sporopollenin polymerization is discussed and suitable model plants for future functional orbicule-research are identified.

Keywords Angiosperms · Orbicules · Pro-orbicule · Sporopollenin · Tapetum · Ubisch bodies

Introduction

Orbicules are readily observable by scanning electron microscopy (SEM) in mature anthers as a layer of tiny particles lining the inner locule wall, in close contact with the pollen grains (El-Ghazaly, 1999; Huysmans et al., 2000; Galati, 2003). Orbicules (syn. Ubisch bodies,[1] con-peito grains) are a-cellular structures of sporopollenin that might

[1]Rowley (1962) coined the term to acknowledge Gerta von Ubisch (1882–1965), a German biologist who did pioneering work on orbicules in the 1920's. She was, however, not the first scientist to describe orbicules; to our knowledge Rosanoff (1865) was the first to discover orbicules.

Electronic supplementary material The online version of this article (doi:10.1007/s12229-014-9135-1) contains supplementary material, which is available to authorized users.

occur on the inner tangential and radial walls of tapetal cells. Usually they are smaller than 1 μm, but orbicules with a diameter up to 15 μm are reported in *Quararibea* (Malvaceae; Nilsson & Robyns, 1974). They originate as lipid droplets (pro-orbicules) within the cytoplasm of tapetal cells, most likely from the rough endoplasmic reticulum (ER; Echlin & Godwin, 1968; Risueño et al., 1969; El-Ghazaly & Jensen, 1986). After exocytosis the pro-orbicules nest on the tapetal plasmalemma and get a sporopollenin coat synchronously with the developing pollen exine (Christensen et al., 1972). The orbicule surface ornamentation often resembles that of the pollen sexine (Rowley et al., 1959; Hesse, 1986; Huysmans et al., 2000), touching the prime challenge in palynology, viz. the source of control over the specific ornamentation of the pollen exine. This resemblance in ornamentation indicates that a similar patterned biosynthesis of sporopollenin is possible on an a-cellular sporophytic structure (pro-orbicule) as on a cellular gametophytic structure (microspore).

The existence of orbicules is known since 1865 when Rosanoff published his observations on anthers of Fabaceae species where he noticed small granules on the inner locule wall that were resistant to concentrated sulphuric acid (Rosanoff, 1865). Both von Ubisch (1927) and von Kosmath (1927) have published lists of species with and without orbicules and considered orbicules to be restricted to taxa with a 'secretory' tapetum type. Ubisch and Kosmath are considered as pioneers in orbicule research, but they were most likely inspired by preceding papers by Chatin (1870; angiosperms), Mascré (1922; Boraginaceae), Schnarf (1923; *Lilium*), and Krjatchenko (1925; *Lilium*). The latter suggested an intratapetal origin for orbicules, possibly in the mitochondria.

Since the early days of orbicule-research, a positive correlation was hypothesised between the presence of orbicules and a parietal tapetum type (von Ubisch, 1927; von Kosmath, 1927), although several species were identified with parietal tapetal cells but lacking orbicules (Huysmans et al., 1998). For a long time only one exception to this hypothesis was known, viz. *Gentiana acaulis* that has anthers with orbicules and an amoeboid tapetum type (Lombardo & Carraro, 1976). Parietal tapeta are the dominant type in land plants and occur in the extant 'basal' angiosperm groups and in most fossil taxa. It is considered as the plesiomorphic condition in angiosperms (Furness & Rudall, 2001a). Parietal tapetum cells keep their individuality and position lining the locules throughout their entire life cycle. In literature, this tapetum type is also referred to as 'secretory' or 'glandular'. In amoeboid tapetal cells, on the contrary, cell walls degenerate prior to fusion of the protoplasts into a plasmodium that invades the locule and assures close contact with the developing microspores. This tapetum type is also known as 'plasmodial'. Next to these two main tapetum types, a few intermediates are described illustrating the complex metabolism of this ephemeral tissue (Pacini et al., 1985). In an invasive tapetum type for instance the cell walls degenerate and the individual protoplasts, without formation of a plasmodium, intrude the locules often in a cyclic pattern during microspore development. The latter type is recorded in a few families throughout angiosperm phylogeny (e.g. Furness & Rudall, 1998, 2001a; Furness, 2008a).

Since mature orbicules consist of sporopollenin they are well represented in the fossil record. One of the few papers devoted to fossil orbicules and tapetal membranes is the ultrastructural work on fossil Pennsylvanian (Carboniferous, 286–320 mya) pollen grains of the Schopfipollenites-type (Taylor, 1976). A more recent study on

Craigia (Malvaceae, Miocene) focused on orbicules and pollenkitt (Zetter et al., 2002). Most data on orbicules in the fossil record are from Cretaceous flowering plants, e.g. the excellent preserved flowers of *Teixeiraea lusitanica* (Ranunculales affinities) with abundant doughnut-shaped orbicules (von Balthazar et al., 2005).

Only a decade ago, it was demonstrated that orbicules occur in all higher order taxonomic units of the angiosperms: from the most early diverged groups (ANITA grade) up to and including the most derived clades of the core-eudicots (see review by Huysmans et al., 1998, updated in Huysmans et al., 2000).

To date our knowledge is insufficient to provide plausible answers to why many plants produce orbicules and why they are absent in several evolutionary successful lineages, even when they are characterized by a parietal tapetum type (e.g. entire family Orchidaceae with >22.000 sp.). Many different functions were hypothesized to explain the occurrence of orbicules (for a review see Huysmans et al., 1998), but none of these is yet satisfactorily proven by experiments or available data. The question whether orbicules have an active function in the anther locules (e.g. in pollen release) or merely represent a by-product of the tapetum, as a reminiscence of the phylogenetically shared origin of tapetum and microsporophytic tissue, remains open.

There is a growing body of literature on the possible allergenic properties of orbicules (e.g. Vinckier & Smets, 2001a, b). Several immunocytological studies provided evidence of localisation of allergens on the orbicular wall (Suàrez-Cervera et al., 2003; Canini et al., 2004; Jato et al., 2010), but negative results were reported for birch (Schäppi et al., 1997; Vinckier et al., 2006). However, the question whether orbicules are dispersed stogether with the pollen grains remains open. Dinis et al. (2007) provided evidence for two grass species showing that the orbicule density of both dehisced and undehisced anthers did not differ significantly.

The study of orbicules could be particularly rewarding since they offer a window on several biological issues on the borderline between gametophyte and sporophyte, such as the ratio of sporophytic and gametophytic genetic control in the development of the sporoderm, the function and chemical composition of lipidic fractions of tapetal origin in flowering plants (Piffanelli et al., 1998), the patterned sporopollenin polymerisation in the anther mediated by 'white lines' and a glycocalyx, and the possible contribution of in vivo self-assembly of sporopollenin in the development of the sporoderm (Gabarayeva & Hemsley, 2006).

Kress (1986: 342) stated that «the original or true function of any character may only be apparent or correctly interpreted in light of its phylogenetic history». In this line of thought we updated the distribution data of orbicules in flowering plants by thoroughly screening the literature since the first review on the topic (Huysmans et al., 1998). Complementary original observations from certain model plants and selected families were added. The current study aims at (1) providing a summary of all data available on orbicule presence/absence in the flowering plants; (2) identifying patterns in the distribution data by mapping them on a recent angiosperm classification; (3) discussing correlations with tapetum types, pollination syndromes and other traits. (4) Finally, the potential of orbicules as a model system for in vivo research on patterned sporopollenin polymerization (including genetic control by gametophyte/sporophyte and self-assembly processes) is demonstrated.

Materials and Methods

Microscopy

In general, mature orbicules are readily visible on the tapetal membrane (remnants of tapetal cells after programmed cell death) and are chemically inert to any preparation method because of their sporopollenin composition. Useful source material therefore also includes herbarium specimens and pickled flowers. Light microscopy is, due to their small size range, less appropriate for orbicule observations (but see Bhandari & Kishori, 1971). Field emission scanning electron microscopy (FE-SEM) is desirable because it provides high-resolution images with negligible electrical charging of the samples at low accelerating voltage.

Dried flowers or anthers were rehydrated for at least 1 h in a wetting agent such as Agepon or Photo Flo (1:200 in distilled water) prior to dehydration by a graded ethanol series (50 %–70 %–95 %–100 %), preferably inside the CPD container(s) to avoid distortion. Pickled flowers were dehydrated completely by continuation of the graded ethanol series from concentration of stock fluid. Living flowers were fixed overnight in FAA (90 ml ethanol 50 %+5 ml acetic acid glacial+5 ml formaldehyde) prior to dehydration. Critical point drying (CPD) involves two washes in 100 % acetone prior to CPD in acetone as intermediary fluid.

Dried anthers were fixed to aluminium stubs with double adhesive carbon tape. If necessary excess pollen grains were gently removed using a cactus spine to clear part of the locule wall. Stubs were sputter coated with platinum (FE-SEM) or gold (SEM). Orbicules were observed using a FE-SEM (Leo supra 55 VP, Zeiss, Jena, Germany) or a SEM (JEOL JSM 6360, Jeol Ltd, Tokyo, Japan) at an accelerating voltage of 5 kV and a working distance of 10 mm.

Literature Search

We have thoroughly screened the scientific literature from January 1997 until December 2012 aiming to provide a state of the art of the distribution of orbicules in angiosperms. Additional papers were found by screening reference lists in consulted works and the palynology reprint collection of the Laboratory of Plant Systematics (KU Leuven, Belgium). Unpublished validated data from lab members and associated collaborators were added as well. Although we aimed to be exhaustive, the resulting dataset might be incomplete because orbicule distribution data has been published in very diverse research fields. Since negative observations require awareness and directed attention, those are most likely underrepresented in many groups.

The data are presented in a phylogenetic order (Appendix S1) following the angiosperm classification by Stevens (2001 onwards). The main arguments for this choice are (1) the accessibility to the entire scientific community by being a free online resource, (2) the fast inclusion of the most recent evolutionary insights in a continuously updated classification, and (3) the presence of genus lists for each family that allows reproducible genus allocation to family level.

For phylogenetic mapping of orbicule distribution data, we used both the APG III topology at order level (APG, 2009) and an updated dahlgrenogram reflecting the relationships between orders in flowering plants (Barthlott, Borsch & Worberg, pers.

com., updated by Worberg). The bubble diagram represents a virtual cross section of 'the tree of life' of angiosperms. Distances between bubbles reflect 'evolutionary distances' between orders or families; bubble size is in relative proportion to species number. A dahlgrenogram does not depict phylogeny in the sense of a branching evolutionary history from inferred hypothesized ancestors. But in the tradition of Rolf Dahlgren (1932–1987), we consider bubble diagrams as powerful tools to depict the distribution of any character in extensive taxa such as angiosperms on a single A4 sheet. Mapping data on both representations was done manually, based on the data in Appendix S1.

Results

Morphology and Localisation of Orbicules

When orbicules are present, they are usually abundant and cover the tapetal remnants on the inner locule surface (Fig. 1a, f). In angiosperms they are seldomly observed adhering to the pollen grains. Size, shape and ornamentation of the orbicular wall may vary between species, e.g. irregular orbicules with a central perforation (Fig. 1h) vs. spherical microechinate orbicules (Fig. 1i). Often the ornamentation of the pollen sexine is similar to that of the orbicules (compare Fig. 1b–c, d–e). Echinate pollen in particular is often reflected in spiny orbicules (Fig. 1i). Orbicules may be more or less embedded in the tapetal remnants (Fig. 1g). When orbicules are absent, the locule wall is smooth and endothecium thickenings may be pronounced (Fig. 1j).

Distribution of Orbicules in Angiosperms

Appendix S1 summarizes the orbicule data at species level with orbicule absence/presence and, if available, the occuring tapetum type. The respective references are added. The data from Huysmans et al. (1998, 2000), covering the period 1865–1996, are included as well, in order to deliver a review as exhaustive as possible. For these entries only cross-reference to Huysmans et al. (1998) or (2000) is provided.

In Table 1 orbicule distribution data are given at family level for each order recognized the in angiosperms sensu APG III (2009). Figure 2 presents a color-coded cladogram at order level. The resulting pattern indicates that orbicules are indeed present all over the topology. Of all orders that have been investigated for orbicules, only five remain without any positive observations: Asterales, Cannellales, Commelinales, Vitales, and Zingiberales. In total 17 orders (Acorales, Arecales, Buxales, Celastrales, Ceratophyllales, Crossosomatales, Dilleniales, Escalloniales, Garryales, Gunnerales, Huerteales, Paracryphiales, Petrosaviales, Picramniales, Santalales, Trochodendrales, Zygophyllales) remain blank spots on the angiosperm orbicule-map because no data on orbicules is available (Fig. 2). In the present study we were able to increase the angiosperm dataset for orbicule presence/absence from 88 to 149 families, an increase of 15 to 36 % of the total number of families since Huysmans et al. (1998); (Fig. 3).

All large (informal) groups in angiosperms such as magnoliids, monocots, basal eudicots, rosids and asterids show a patchy image with both positive and negative

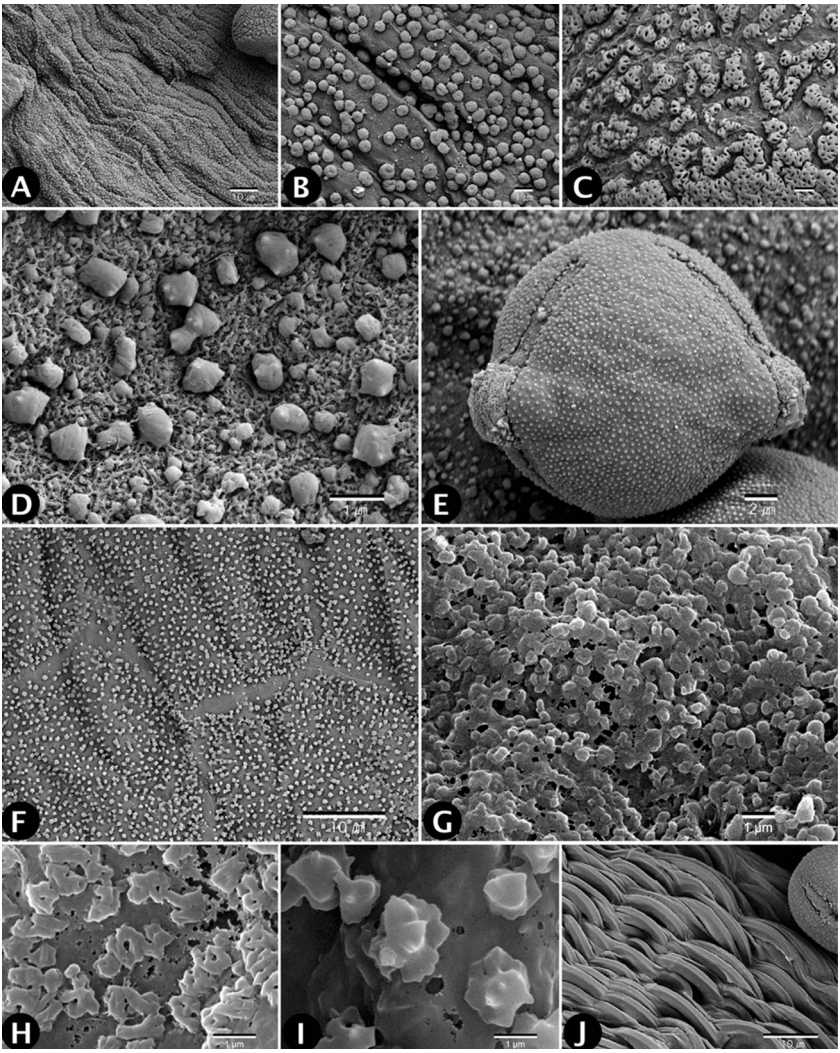


Fig. 1 General features and morphology of orbicules in angiosperms. Collections observed are specified indicating location: BR = National Botanic Garden Belgium, E = herbarium of Royal Botanical Gardens Edinburgh, RBGE = Royal Botanical Gardens Edinburgh, U = herbarium of Naturalis Biodiversity Center, Utrecht University. **a**–**c** *Chlorophytum alismaefolium* Baker (living material, cultivated in RBGE; Agavaceae; FE-SEM). **a** General view on inner locule wall densely covered with orbicules. In top right corner part of a pollen grain visible to allow size comparison. **b** Detail of orbicules; note one up to several perforations in orbicular walls. **c** Detail of pollen wall at aperture border showing sexine elements randomly dispersed on apertural membrane with similar ornamentation pattern as orbicules. **d**–**e** *Solanum virginianum* L. (Hedge et al. 7412, E; Solanaceae; FE-SEM). **d** Spiny orbicules regularly spaced on the inner locule wall, which is covered with a network-like structure. **e** Pollen grain with similar microechinate sexine ornamentation. **f** *Hordeum vulgare* L. (Davis 49564, E; Poaceae; FE-SEM). View on inner locule wall evenly covered with orbicules. Note pattern of lines without orbicules, possibly indicating the outlines of the former tapetal cells. **g** *Polyalthia subcordata* (Bl.) Bl. (van Balgooy & van Setten 5667, U; Annonaceae; SEM). Dense layer of orbicules, partly fused with tapetal membrane and interconnected by threads. **h** *Didymosalpynx abbeokutae* (Hiern) Keay (Hart 851, BR; Rubiaceae; SEM). Irregular shaped orbicules with perforations. **i** *Cubanola domingensis* (Britton) Aiello (living material, cultivated in BR; Rubiaceae; SEM). Conspicuous spiny orbicules. **j** *Boopis graminea* Phil. (Gardner et al. 4635, E; Calyceraceae; FE-SEM). Orbicules are absent; the inner locule wall is smooth. Note the ridges of the endothecium

Table 1 Orbicule distribution data for flowering plants summarized at order level (sensu APG III) based on data in Appendix S1

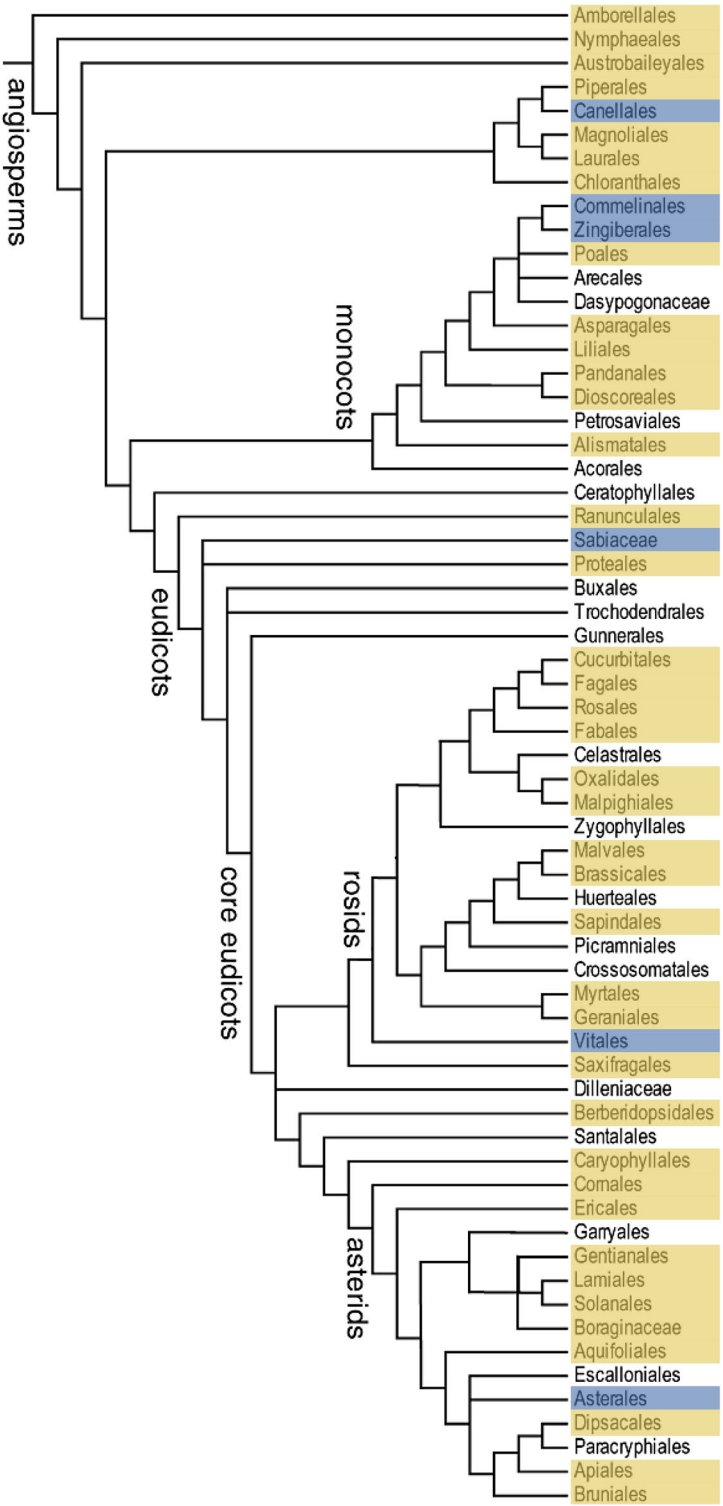
Order	Total # fam.	# fam. +	# fam. +/–	# fam. –	# fam. no data
Amborellales	1	1			
Nymphaeales	3	2			1
Austrobaileyales	3	2			1
Chloranthales	1	1			
Magnoliids					
Cannellales	2			1	1
Piperales	5	3			2
Laurales	7	2	1	1	3
Magnoliales	6	3	1	1	1
Monocots					
Acorales	1				1
Alismatales	13	2		1	10
Petrosaviales	1				1
Dioscoreales	5		2	1	2
Pandanales	5	2		3	
Liliales	10	4			6
Asparagales (incl. *Orchidaceae*)	14	6		1	7
Commelinids					
Arecales	1				1
Commelinales	5			2	3
Poales	16	5			11
Zingiberales	8			3	5
Possible sister of eudicots					
Ceratophyllales	1				1
Eudicots					
Ranunculales	7	2	1	1	3
Proteales	3	2			1
Sabiaceae	1			1	
Trochodendrales	1				1
Buxales	2				2
Core eudicots					
Gunnerales	2				2
Dilleniales	1				1
Saxifragales	14	3			11
Rosids					
Vitales	1			1	
Fabids					
Zygophyllales	2				2
Celastrales	2				2
Oxalidales	7	1			6
Malpighiales	36	3	4		29

Table 1 (continued)

Order	Total # fam.	# fam. +	# fam. +/−	# fam. −	# fam. no data
Cucurbitales	7	1		1	5
Fabales	4	1	1		2
Fagales	7	2			5
Rosales	9	4		1	4
Malvids					
Geraniales	3	1	1		1
Myrtales	9	1		1	7
Crossosomatales	7				7
Picramniales	1				1
Huerteales	3				3
Brassicales	17	3		5	9
Malvales	10	1	1		8
Sapindales	9	2			7
Berberidopsidales	2	1			1
Santalales	7				7
Caryophyllales	34	6	2	1	25
Asterids					
Cornales	6	4	1	1	
Ericales	22	5	1	1	15
Lamiids					
Garryales	2				2
Gentianales	5	2	3		
Lamiales	23	7	3	2	11
Solanales	5	1	1	1	2
Campanulids					
Aquifoliales	5		1		4
Asterales	11			3	8
Escalloniales	1				1
Bruniales	2	1			1
Paracryphiales	1				1
Dipsacales	2	1		1	
Apiales	7	1			6
Unplaced families	8	1			7
Total	416	90	24	35	267

fam. number of families, + orbicules present, − orbicules absent, +/− orbicules absent or present

Fig. 2 Distribution of orbicules in angiosperms at order level. Cladogram of angiosperms (modified from APG III) reflecting the hypothesized relationships between orders, color-coded for presence of orbicules (based on data in Appendix S1). Orders with at least one species with orbicules present are indicated in *yellow*, orders with only negative observations are marked in *blue* ▶


Amborellales
Nymphaeales
Austrobaileyales
Piperales
Canellales
Magnoliales
Laurales
Chloranthales
Commelinales
Zingiberales
Poales
Arecales
Dasypogonaceae
Asparagales
Liliales
Pandanales
Dioscoreales
Petrosaviales
Alismatales
Acorales
Ceratophyllales
Ranunculales
Sabiaceae
Proteales
Buxales
Trochodendrales
Gunnerales
Cucurbitales
Fagales
Rosales
Fabales
Celastrales
Oxalidales
Malpighiales
Zygophyllales
Malvales
Brassicales
Huerteales
Sapindales
Picramniales
Crossosomatales
Myrtales
Geraniales
Vitales
Saxifragales
Dilleniaceae
Berberidopsidales
Santalales
Caryophyllales
Cornales
Ericales
Garryales
Gentianales
Lamiales
Solanales
Boraginaceae
Aquifoliales
Escalloniales
Asterales
Dipsacales
Paracryphiales
Apiales
Bruniales
angiosperms
monocots
eudicots
core eudicots
rosids
asterids

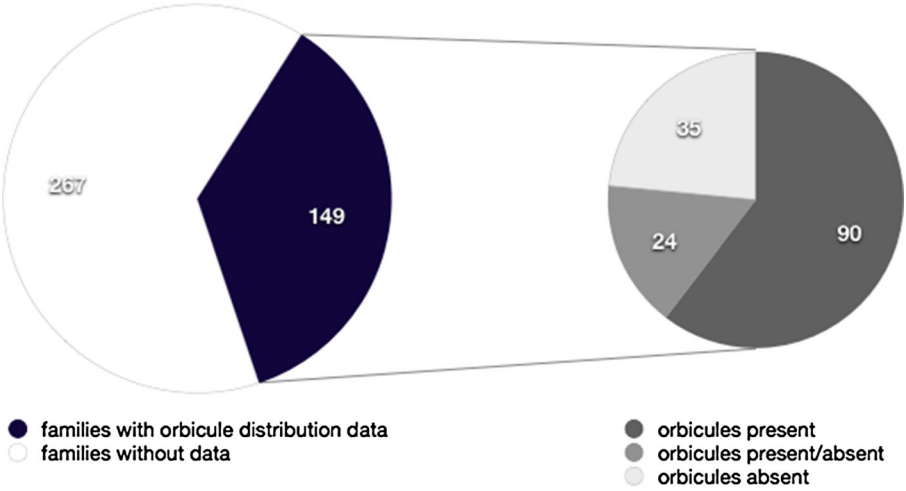


Fig. 3 The present survey includes orbicule distribution data for 149 families of which 90 families only have positive observations and 35 only negative. In merely 24 families both presence and absence of orbicules was recorded

observations. At family level, however, less variation is encountered: only 24 families (16 % of all families studied) show both presence and absence of orbicules between their representatives, viz. Annonaceae, Apocynaceae, Aquifoliaceae, Berberidaceae, Cactaceae, Convolvulaceae, Dioscoreaceae, Euphorbiaceae, Fabaceae, Gentianaceae, Lamiaceae, Linaceae, Loasaceae, Malvaceae, Melianthaceae, Monimiaceae, Nartheciaceae, Oleaceae, Passifloraceae, Plantaginaceae, Polygonaceae, Rubiaceae, Salicaceae and Tetrameristaceae (Table 1). Within these groups, orbicule distribution data generally are consistent at generic level. Only 8 genera deviate from this pattern: *Aletris* (Nartheciaceae), *Coptosapelta* (Rubiaceae), *Dioscorea* (Dioscoreaceae), *Gentiana* (Gentianaceae), *Ilex* (Aquifoliaceae), *Ipomoea* (Convolvulaceae), *Monodora* (Annonaceae), and *Passiflora* (Passifloraceae).

In order to visualise the phylogenetic signal inherent in the distribution data at order level compiled in Table 1, we used the bubble diagram by Barthlott, Borsch and Worberg (pers. com., updated by Worberg, Fig. 4). The total absence of orbicules in Orchidaceae (here depicted separately from Asparagales) is conspicuous. However, (negative) data are extremely rare in this family and therefore orchids represent a target group for further studies.

Discussion

Orbicules: Valuable New Systematic Character?

Orbicule production by tapetal cells in a secretory metabolic phase can be interpreted as a primitive, possibly neotenic feature in flowering plants since orbicules occur also in bryophytes, pteridophytes and gymnosperms. However, data in these groups are highly fragmented. Orbicules are omnipresent in Gnetales: *Ephedra* (El-Ghazaly & Rowley,

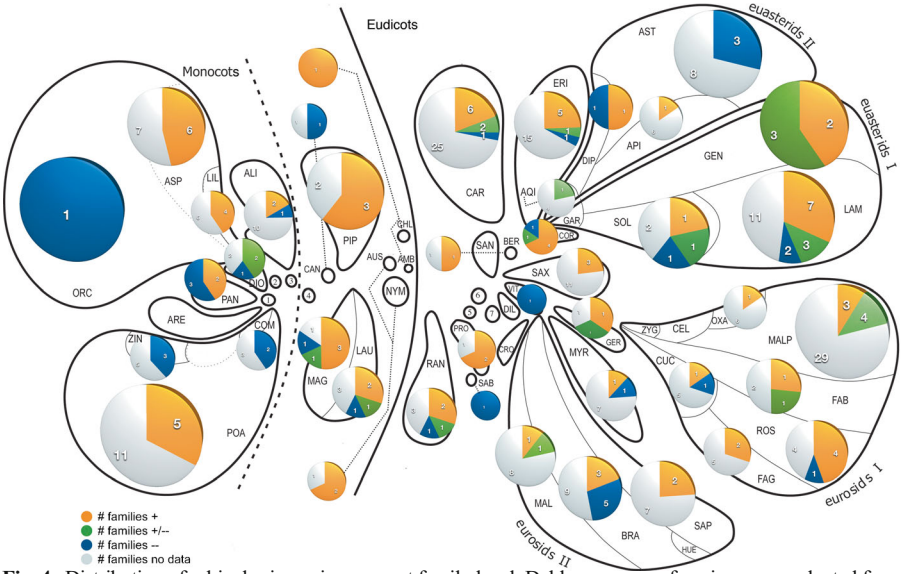


Fig. 4 Distribution of orbicules in angiosperms at family level. Dahlgrenogram of angiosperms, adapted from Barthlott, Borsch and Worberg (unpubl.), updated by A. Worberg, (pers. com.). *Bubbles* represent orders or clusters of related orders; their size is relative to the number of species. Orders are labeled by three letter acronyms except for Malpighiales (MALP) to distinguish it from Malvales (MAL). Bruniales, Escalloniales, Paracryphiales and Picramniales are not represented on the figure. Orders are listed in Table 1 for reference. Pie diagrams for each order summarize number of families with orbicules (*yellow*), without orbicules (*blue*), both with and without orbicules (*green*), and without data (*greyish white*) respectively. For Orchidaceae (see Discussion) and Sabiaceae (position uncertain) a pie diagram on family level was added. Notes: 1 = Petrosaviales, 2 = Acorales, 3 = Ceratophyllales, 4 = Trochodendrales, 5 = Buxales, 6 = Gunnerales

1997; Doores et al., 2007), *Gnetum* (Carniel, 1966), and *Welwitschia* (Zavada & Gabarayeva, 1991). *Cryptomeria japonica*, a coniferous species in Taxodiaceae and a major cause of pollinosis in Japan, produces orbicules (e.g. Hosoo et al., 2005). Audran (1981) described orbicule development in *Ceratozamia* (Cycadaceae) and Rowley and Walles (1987) in *Pinus*. Blackmore et al. (2000) reported orbicules in 13 genera of pteridophytes, but see Alarid et al. (2005) for conflicting data on *Isoetes*. The majority of pteridophytes are shown to have an amoeboid tapetum (Parkinson & Pacini, 1995), however, many homosporous ferns have globular bodies in their sporangial locules that were considered to be homologous to orbicules in spermatophytes (Lugardon, 1981). In bryophytes and lycopods, on the contrary, a parietal tapetum is omnipresent (Pacini et al., 1985) and similar structures to orbicules are recorded, but their homology remains unresolved due to lack of data.

As such the overall systematic value of orbicules in flowering plants is restricted for not being an apomorphic feature. However, mere orbicule presence/absence data reveal an interesting pattern in angiosperms (Appendix S1, Fig. 2). In total 149 out of 416 families (36 %) are represented in Appendix S1 with orbicule data for at least one species. Both orbicule presence and absence is recorded from the earliest diverging flowering plants up to most recent diversified asterid clades. All large (informal) groups in angiosperms such as magnoliids, monocots, basal eudicots, rosids and asterids show a patchy image with both positive and negative observations. At family level, however, less variation is encountered: only 24 families (16 % of families studied) show both

absence and presence of orbicules between their representatives (see Results). The majority of families is thus surprisingly constant for orbicule data, providing a predictive value that surely has potential for systematically oriented research questions. In Brassicaceae, for instance, orbicules were never recorded; also in *Arabidopsis thaliana* they are absent, despite its parietal tapetum (Murgia et al., 1991). However, Staiger et al. (1994), in their immunocytochemical study on *Sinapis alba*, provided TEM pictures of «globules resembling pro-Ubisch bodies which appeared at tetrad stage» labelled with two tapetum specific proteins. According to the authors, the pro-orbicules contain sporopollenin precursors but receive no polymerized sporopollenin wall later in development (Staiger et al., 1994). Lamiaceae is a well-studied family generally lacking orbicules on the inner locule wall. All positive observations in Appendix S1 represent former Chloanthaceae species (Ray & El-Ghazaly, 1987), a family that was merged with Verbenaceae of which many taxa were recently accomodated in Lamiaceae (Harley et al., 2004). On a lower level of classification, we see that orbicule distribution data are generally consistent at generic level. The observation of both presence and absence in a single genus is very rare and is only found in 8 genera (see Results). In Rubiaceae for example, the most thoroughly studied angiosperm family for orbicules, intrageneric variation was observed and described in only one (*Coptosapelta*) out of 163 genera investigated (Verellen et al., 2004; Verstraete et al., 2011). A discussion on this variation can be found in Huysmans et al. (2010) for *Monodora* (Annonaceae) and in Verstraete (2009) for the other genera. These deviant genera are interesting groups for further study into orbicule distribution and tapetal characteristics.

Recent observations in Annonaceae showed that orbicules are much more common in the family than previously perceived (Huysmans et al., 2010). Moreover, an unequivocal phylogenetic pattern appeared by plotting the available orbicule distribution data on the most recent family phylogeny. In *Anaxagorea*, basal clade and sister to all other Annonaceae, Ambavioideae and the 'short branch clade', orbicules were recorded. In the most derived 'long branch clade' orbicules are consistently absent (*Monodora crispata* being the single exception). A recent study in Rubiaceae that investigated the phylogenetic signal of orbicules also demonstrated an evolutionary trend towards the absence of orbicules (Verstraete et al., 2011). The same trend emerges at angiosperm level based on our collective data: orbicules are common in the ANITA-grade and 85 % of the monocots studied produce orbicules, with Orchidaceae, Commelinales and Zingiberales as notable exceptions. Within eudicots the asterids are most densely sampled with 61 % orbicule presence. Asteraceae and the majority of Lamiaceae lack orbicules. These observations indicate firstly that orbicules are found all over the topology of flowering plants and, secondly, that an evolutionary trend exists towards orbicule absence in more derived clades.

Orbicules come in a wide array of different sizes, shapes and densities (Fig. 1). Do any of these features provide additional potential systematic characters? Orbicule characters are systematically analysed in several taxa of Gentianales such as Gentianaceae (Vinckier & Smets, 2003), Apocynaceae s.l. (Vinckier & Smets, 2002c), Loganiaceae s.l. (Vinckier & Smets, 2002a), and Rubiaceae (Huysmans et al., 1997; Vinckier et al., 2000; Verstraete et al., 2011). Overall conclusion for Gentianales was that orbicules are a common feature and that morphological characteristics might be useful at tribal level (Vinckier & Smets, 2002b).

The key issue here is the character stability at species level. Very few studies have paid attention to the intraspecific variability of orbicule characters; mostly only a single specimen per species is investigated. Verellen et al. (2004) observed five specimens of *Coptosapelta tomentosa* (Rubiaceae) and found three different orbicule types in different specimens and one specimen without orbicules. Orbicules are absent in most Lamiaceae and for some species such as *Heterolamium debile*, *Melissa officinalis*, *Prunella vulgaris*, *Stachydeoma graveolens* and *Thymbra spicata*, this negative observation was confirmed in more than two specimens (Moon et al., 2008b). A detailed developmental study at ultrastructural level of *Anaxagorea brevipes* (Annonaceae) showed that the parietal tapetum reluctantly invades the locule at the early tetrad stage and secretes both orbicules and other globular lipoidal concretions (Gabarayeva, 1995). The variety of size and chemical composition of these tapetal cytoplasmic globular inclusions reflects the variety of the tapetal functions according to Gabarayeva (1995).

Correlation Orbicules-Tapetum Type

It is impossible to separate orbicules and the tissue where they originate if it comes to explain their form and function. At present, identification keys are available to distinguish between tapetum types (e.g. Pacini, 1997). The various types, however, can be reduced to two basic types that were already recognised by Goebel (1901): (1) parietal (syn. secretory or glandular) tapetum and (2) amoeboid (syn. plasmodial or intrusive) tapetum. An intermediate type, (3) invasive tapetum, is sometimes mentioned. In this third type, the cell walls degenerate and the individual protoplasts, without formation of a plasmodium, intrude the locules often in a cyclic pattern during microspore development. The latter type is recorded in a few families throughout angiosperm phylogeny (e.g. Furness & Rudall, 1998, 2001a; Furness, 2008a). Ultrastructural research of tapetal cells during their entire development, an element of major importance, revealed the highly dynamic nature and amazing morphological and cytological variation of this specialized nutritious tissue (e.g. Rowley et al., 1992; Rowley, 1993). For the great majority of plants no detailed information on tapetum type is available. When Davis (1966) compiled embryological data for the angiosperms, information on the tapetum type (amoeboid or parietal) was available for only 231 families. Pacini et al. (1985: table 2) updated her results and stressed that, astonishingly, the tapetum type was not investigated in almost half of the angiosperm families.

The positive correlation between orbicules and a parietal tapetum type is hypothesized since almost a century. Our present results confirm this general pattern statistically (Table 2). However, we also detected several additional species with a non-parietal tapetum type and orbicules (Table 3). *Tradescantia virginiana* (Commelinaceae), for example, has an amoeboid tapetum that produces tapetal derived granules but with less obvious sporopollenin accretion on their surface and smaller in size than average orbicules (Tiwari & Gunning, 1986c). The granules were considered analogous to the tapetal pro-orbicules of parietal tapeta by these authors. A highly interesting observation concerns *Sauromatum venosum* (Araceae), which has inaperturate pollen grains with an endexine and spines, both polysaccharidic in nature (Weber et al., 1998). Remarkably, orbicule-like structures occur, also polysaccharidic in composition (tested with PAS-reaction for detection of neutral polysaccharides). Both PAS-positive spines and orbicule-like structures in *Sauromatum* originate from the amoeboid tapetum and

Table 2 Summary of the numbers of species with information on tapetum type and presence/absence of orbicules (based on data in Appendix S1). Only taxa where the observations are unambiguous are taken into account

	Orbicules present	Orbicules absent	Total
Parietal tapetum	211 (87.2 %)	31 (12.8 %)	242
Amoeboid tapetum	7 (20 %)	28 (80 %)	35
Invasive tapetum	4 (36.4 %)	7 (63.6 %)	11
Total	222	66	288

are formed synchronously (during first pollen mitosis) with an identical pattern formation (Weber et al., 1998). In Asteraceae different tapetum types occur with several intermediate forms (reviewed by Pacini, 1996), while orbicules appear to be consistently absent throughout the entire family and also in sister clades Calyceraceae and Goodeniaceae (Appendix S1). Therefore Asteraceae might provide an interesting case for increasing our understanding of the relationship between tapetum metabolism and orbicule production. An ontogenetic study of pollen and anthers of *Cabomba caroliniana* (Cabombaceae; Taylor et al., 2008) indicated the presence of an amoeboid

Table 3 Species with an amoeboid or invasive tapetum type that are reported to produce orbicules

Family	Species	Reference
Amaranthaceae	*Beta vulgaris*	Huysmans et al., 1998; Furness, 2008a
Annonaceae	*Anaxagorea brevipes*[a]	Gabarayeva, 1995
Apocynaceae	*Vinca rosea*[a]	El-Ghazaly & Nilsson, 1991
Asteraceae	*Cosmos bipinnatus*	Blackmore & Barnes, 1985
Bromeliaceae	*Aechmea dichlamydea* var. *trinitensis*[a]	Sajo et al., 2005
Butomaceae	*Butomus umbellatus*[b]	Fernando & Cass, 1994
Cabombaceae	*Cabomba caroliniana*	Taylor et al., 2008
Commelinaceae	*Tradescantia virginiana*[b]	Tiwari & Gunning, 1986c
Fabaceae	*Acacia conferta*	Kenrick & Knox, 1979
	Acacia iteaphylla	"
	Acacia subalata	"
Fagaceae	*Quercus robur*	Rowley & Gabarayeva, 2004
Gentianaceae	*Canscora decussate*[b]	Vinckier & Smets, 2003
	Gentiana acaulis	Lombardo & Carraro, 1976
	Swertia perennis[b]	Vinckier & Smets, 2003
Lauraceae	*Persea palustris*[b]	Furness & Rudall, 2001a
Malvaceae	*Abutilon pictum*	Strittmatter et al., 2000
	Modiolastrum malvifolium	Galati et al., 2007
Nymphaeaceae	*Nymphaea colorata*[a]	Rowley et al., 1992
	Nymphaea mexicana[a]	Gabarayeva & El-Ghazaly, 1997

[a] Tapetum type is specified as cyclic invasive

[b] Species show very small sporopollenin granules, homology with orbicules is not clear

tapetum. The degree of migration of tapetal cells into the locules, however, was variable between anthers and the secretory function was conserved. The latter is expressed in the presence of orbicules. These observations highlight the character plasticity present in basal angiosperms and support the conclusion put forward by Furness and Rudall (2001a) that characters present in these taxa potentially represent evolutionary experimentation in early angiosperm lineages.

Finally, it should be noted that the presence of a parietal tapetum is not the single determinative proxy to find orbicules. Of the 242 species that are indicated in Appendix S1 as having a parietal tapetum, 31 species (=12.8 %) lack orbicules (Table 2). This rough count does not include Orchidaceae, with over 22.000 species, which are believed to be characterized by a parietal tapetum (Pacini, 2009). Orbicule data are extremely scarce for orchids, possibly because negative observations require directed attention. *Doritis* is actually the only orchid genus where orbicules were reported, but we seriously doubt this interpretation judging their figures 8 and 12 (Wolter et al., 1988).

Correlation Orbicules-Pollination Syndrome

In their analytical key for tapetum types Pacini et al. (1985) correlated the occurrence of orbicules with the presence of pollenkitt, that is with the pollination syndrome. Pacini (1997) suggested that orbicules are absent in species with a strictly entomophilous pollination in which pollenkitt is present, and that they only occur in anemophilous species (without pollenkitt) and entomophilous angiosperms with a non-specific pollination syndrome: «Only few taxa with a parietal tapetum with a strong entomophilous syndrome, such as the pumpkin (*Cucurbita pepo*) and orchids lack Ubisch bodies entirely» (Pacini & Franchi, 1993: 5). Our new data do not contradict this statement. Other examples of taxa that have a parietal tapetum but lack orbicules are found in Brassicaceae and Balsaminaceae. These two families, together with Orchidaceae, are therefore very suitable for future studies on the correlation between orbicules and pollination syndrome.

Orbicules have not been observed in taxa with viscin threads, elastoviscin, massulae or compact pollinia (Pacini, 1997). On the other hand, they are present in *Acacia* species that develop polyads (Kenrick & Knox, 1979; G. Prenner, pers. com.).

Orbicules as a Model System for Sporopollenin Polymerisation

Orbicules provide an interesting model to study sporopollenin biosynthesis since they are a-cellular structures, independent of cytoplasmic control, contrary to the pollen exine (Clément & Audran, 1993). Very little experimental work has been done concerning the factors controlling orbicule formation. The pioneering work on *Canna* (Tiwari & Gunning, 1986a) and *Tradescantia* (Tiwari & Gunning, 1986b, c, d), both with amoeboid tapetum lacking orbicules, deserves special mention. The authors used colchicine treatments to investigate the role of cortical microtubules in the developing invasive tapetum. In *Tradescantia* the treatments prevented cell fusion. In both species investigated there was a disordered deposition of sporopollenin on all available extracellular lipidic surfaces and also on the outside of tapetal plasma membranes. The effect of colchicine provided evidence that amoeboid tapeta do participate in the synthesis and

secretion of sporopollenin even though this activity is only manifest after experimental disturbance. Tiwari and Gunning (1986d) suggested that the availability of lipidic surfaces and extracellular space imposes physical constraints on the amount of sporopollenin deposited at any particular site.

Rowley et al. (1959: 537) accurately pointed out already half a century ago that «… their [orbicules] morphology, composition, and position outside of the pollen wall seem to touch upon the prime problem of pollen morphology; i.e., the source of control over the specific ornamentation of the exine». The question as to whether the wall around the haploid microspore, and especially its ornamentation or patterning, is controlled by the microspore itself or by the diploid, sporophytic tapetum has long challenged angiosperm palynologists (reviewed by Blackmore et al., 2007) and has not yet been fully resolved. Pro-orbicules, after exocytosis and generally during tetrad stage, nest on the tapetal plasmalemma, and it is on this lipidic surface that they receive their sporopollenin coat. Moreover, the mode of development seems to be similar between exine and orbicular wall (Christensen et al., 1972). Evidence is growing that orbicule wall development also involves a glycocalyx-template and white line centred lamellae were observed in sub-mature orbicules of *Rondeletia odorata* (Rubiaceae; S. Huysmans, unpubl. data) reflecting the process of endexine development in the same species (El-Ghazaly et al., 2001).

The study of taxa with exineless pollen might yield interesting information concerning the potential of sporopollenin production by the tapetum. Pollen grains possessing a much-reduced exine and elaborated intine (omniaperturate and/or exineless) are known to occur in 54 families of angiosperms and are nearly ubiquitous in Zingiberales (Kress, 1986). For this study we have observed *Etlingera* (Zingiberaceae) with exineless pollen grains and without orbicules. In the large genus *Xylopia* (Annonaceae) at least four species have sporopollenin-lacking pollen (Tsou & Johnson, 2003) and no orbicules. It is noteworthy that in two species of *Xylopia* in which the tapetum starts to degenerate before meiosis, the pollen does not develop a typical exine wall (Tsou & Johnson, 2003).

Origin of Orbicules and Tapetal Lipid Metabolism

Insight in the complex lipid metabolism of tapetal cells appears to be crucial in our understanding of the distribution and function of orbicules in angiosperms. Pro-orbicules are indeed simple lipid vesicles originating from the endoplasmic reticulum in the tapetal cytoplasm (see Staehelin, 1997 for a review of ER functional domains in plant cells), while the sporopollenin wall of mature orbicules is considered as a complex mixed polymer of acyl lipid precursors and phenyl-propanoids (Scott, 1994). Piffanelli et al. (1998) reviewed the biogenesis and function of four lipidic structures associated with male gametophytes: exine and pollenkitt as extracellular lipidic structures, and storage oil bodies and a dense membrane network as intracellular lipidic structures. The first two are mostly controlled by the sporophytic genome, while the other two are primarily regulated by the gametophytic genome. Piffanelli et al. (1998) mentioned Ubisch bodies as one of the lipidic bodies produced by the anther in Brassicaceae (their Fig. 1a), which is in conflict with our present results since there are no reports of orbicules in Brassicaceae (but see Staiger et al., 1994). Tapetal cell degradation appears to involve apoptosis-like programmed cell death, which indicates

a controlled process (Parish & Li, 2010). Therefore we prefer the term tapetum maturation above degeneration for this process since the long persistence of the tapetal mitochondria indicate the necessity for an energy supply, confirming that it not simply concerns necrosis (Papini et al., 1999). Wu et al. (1997) described a novel class of lipid containing organelles in the tapetum of *Brassica* that they termed tapetosomes (but see Dunbar, 1973). Whether the initiation or chemical composition of tapetosomes or elaioplasts (Piffanelli & Murphy, 1998) is related to pro-orbicules is unknown.

Evo-Devo and Orbicule Marker Genes

Evolutionary developmental approaches have greatly expanded our understanding of the genetic pathways that are involved in pollen wall development and the rate of gametophytic and sporophytic factors in the patterned polymerisation of sporopollenin on a lipidic interface, i.e. the plasmalemma of the microspores (reviewed by Wilson & Zhang, 2009). Evo-devo studies on model plants reveal an increasing number of genes expressed in the tapetum that when silenced (or in natural mutants) cause male-sterility or an arrest of the wild type microspore development (e.g. Goldberg et al., 1993; Kapoor et al., 2002; Yui et al., 2003; Ariizumi et al., 2004; Suzuki et al., 2008; Wu et al., 2008; McNeil & Smith, 2010; Zhang et al., 2010; Li et al., 2011). In rice, two putative orbicule marker genes, *Os Raftin1* and *Os Raftin2*, are downregulated in *Wax-deficient anther1* (*wda1*) anthers that are lacking orbicules contrary to the wild-type (Jung et al., 2006). The rice *RAFTIN* genes are homologs of the wheat *RAFTIN1* gene located at orbicules and exines, and supposed to have a guiding role in the proper fixation of sporopollenin polymers in the exine (Wang et al., 2003). Jung et al. (2006) concluded that the downregulation of both orbicule marker genes and the absence of orbicules and cytoplasmic lipid bodies in *wda1* anthers imply that the *wda1* mutant possibly affects the transfer of sporopollenin from the tapetum to the pollen walls via these organelles. Our results strongly question this transport function attributed to orbicules because firstly not all species with a parietal tapetum produce orbicules (Appendix S1), and secondly to date no enzyme could be characterized that is able to depolymerise sporopollenin. Thom et al. (1998) provided experimental evidence for reaggregation of materials obtained after fractionation of dissolved sporopollenin. To our knowledge there is no evidence available for reaggregation of sporopollenin *in vivo*. Moreover, orbicules generally remain associated with the tapetal plasmalemma throughout their development and thus active movement of orbicules is restricted to intrusion into the locules of invasive parietal tapetal cells that maintain their individual protoplasts (e.g. in *Anaxagorea*: Gabarayeva, 1995; *Nymphaea*: Gabarayeva & El-Ghazaly, 1997 and Rowley et al., 1992; *Rondeletia*: S. Huysmans, unpubl. data). Cyclic invasion of secretory tapetal cells into the locular space might be a much more common feature than presently considered (Gabarayeva et al., 2009).

Self-Assembly Processes and Patterning

There is growing evidence on the importance of self-assembly processes in pollen wall development. Gabarayeva and Hemsley (2006) summarized the developmental facts during tetrad and microspore stages and suggested mechanisms of molecular interaction to explain the wide range of variation in ornamentation patterns on angiosperm pollen

grains. The authors concluded that in the sequence of exine development four main stages can be recognized, each with a different mechanism for wall construction: (1) formation of glycocalyx by self-assembly of micelles, (2) insertion of sporopollenin receptors under control of genome, (3) accumulation of receptor-dependant sporopollenin under control of sporopollenin receptors and (4) accumulation of receptor-independent sporopollenin by self-assembly. Exine pattern determination was originally attributed to callose (e.g. Blackmore & Barnes, 1990), however, genomic control of glycocalyx construction components is the most likely method by which genetic control of initial patterning is exerted (Gabarayeva & Hemsley, 2006). Evidence exists that during the middle tetrad stage sporopollenin can be produced from both tapetum and microspore although at present there is no data to suggest whether sporopollenin from these two sources differs in chemistry. If sporopollenin monomer is to be derived from the microspore, a site of production and a mechanism of transport are required. Droplets of lipid-like material synthesised in the interior of the ER membrane (Staehelin, 1997) are likely candidates for the monomers while the white-line-centred lamellae (see Scott, 1994) may well provide an appropriate transport conduit (Gabarayeva & Hemsley, 2006). During late tetrad stage sporopollenin deposition continues while callose is disintegrated by callase activity, but the source of this sporopollenin has yet to be determined and may be from either the microspore or the tapetum. The disintegration of callose may facilitate the penetration of sporopollenin precursors from the tapetum. In the subsequent free microspore stage sporopollenin is almost certainly derived from the tapetum and the mechanism of accumulation is largely self-assembly. Consequently the ultimate pattern results from autopolymerisation of bulk monomer (Gabarayeva & Hemsley, 2006 and references therein).

If self-assembly processes interfere with the work of the genome in pattern determination of the sporoderm, we could hypothesize parallel mechanisms for the sporopollenin polymerisation and patterning on the pro-orbicule core. The gametophytic genomic component and a callose wall are missing in the orbicule model and yet a patterned accumulation of sporopollenin with similar ornamentation to the pollen wall (Hesse, 1986; Huysmans et al., 1998) is still achieved.

Function(s) of Orbicules

One of the most intriguing open questions about orbicules is their function. Although many hypotheses on functions attributed to orbicules can be found in the literature, none of them is yet satisfactorily proven (reviewed in Huysmans et al., 1998). Two opposing lines of thought can be distinguished: orbicules play an active role or are just a by-product.

One hypothesis is that the tapetum has the vestigial capacity to polymerize sporopollenin because it shares a phylogenetically identical origin with the sporogenous tissue (Hesse, 1986). Orbicules are just mere by-products of the tapetal cell metabolism. This explains the presence of orbicules in unrelated taxa, the similarities in ornamentation between pollen and orbicules of the same species, and also the absence of orbicules in species with an amoeboid tapetum. Amoeboid tapeta may have lost the capacity to polymerize sporopollenin during evolution. The selective pressure for the evolution of absence of orbicules could be the conservation of resources, viz. sporopollenin precursors. However, the classic model of anther development involving three

'germ layers' that give rise to specific cell lineages (L1 to epidermis, L2 to endothecium, middle layers, outer tapetum and archesporial cells, and L3 to connective and inner tapetum) and the well accepted dual origin of tapetum in angiosperms, has been challenged. Tsou and Johnson (2003) showed that tapetum differentiation in Annonaceae may instead be induced by chemical signaling from neighbouring sporogenous cells, and that ontogenetic origin has little or no significance for tapetum formation. Tapetum differentiation might be position-dependent rather than cell autonomous (Tsou & Johnson, 2003).

Another hypothesis is that orbicules participate actively in sporoderm formation by representing a transport system of sporopollenin between the tapetum and the developing microspores. This idea is based on observations of connections between orbicules and pollen sexines, mainly in grasses, or observations of a close contact between microspores and orbicules in other species. However, orbicules do not erode nor are they eliminated during microspore development, on the contrary their sporopollenin coat grows synchronously with the pollen exine (see Christensen et al., 1972 for comparative data on *Sorghum*). Moreover, so far no enzyme has been found to depolymerize sporopollenin. Since orbicules are not universally present, not even in species with a parietal tapetum, it is unlikely that they have such a general and crucial function as exine construction. Another active role for orbicules could be contributing to pollen dispersal. Orbicules could form a hydrophobic locule surface from which pollen can easily detach (Heslop-Harrison, 1968). Or, because exine and orbicules both consist of sporopollenin, they carry the same electrical charge and therefore repel one another (Pacini & Franchi, 1993). Orbicules could even present a reward for pollinators and therefore help in the attraction of visiting animals (Huysmans et al., 2000). But what about plant species that lack orbicules? Their pollen is being dispersed as well, without the presence of a hydrophobic surface or a repelling force. Orbicules are also very common in anemophilous species where offering a floral reward would be superfluous. Until now, there is no evidence of a correlation between orbicules and a particular pollination syndrome.

Conclusions and Future Directions

Orbicules might represent one of the last 'secrets of the anther', however, we demonstrated that they are actually commonly occurring in all higher order taxa of flowering plants and that they have great potential as model system to increase our knowledge in some fundamental issues in palynology and cell biology. Our results identified families and orders that are lacking any orbicule data. Orchidaceae, Arecales, rosids in general with Malpighiales in particular are 'hot' taxa for future morphological studies. Poaceae (rice, wheat, corn,…) and Solanaceae (tomato, tabacco,…) are plausible candidates for directed evo-devo approaches. Many new developments and data can be expected if functional genetic experiments on model plants, set up to identify the genes and gene products that control sporopollenin polymerisation and patterning, take both microspores and orbicules into account when screening phenotypes. We hope this review might raise awareness of orbicules and inspire a new generation of molecular biologists, palynologists, and systematists alike to explore the potential of orbicules in their own field of research.

Acknowledgments The authors gratefully thank N. Geerts (KU Leuven) and F. Christie (RBG Edinburgh) for technical assistance, A. Wortley and A. Poulsen for hospitality and sampling assistance to the last author at Royal Botanic Gardens Edinburgh, and A. Worberg for drawing an updated and tailor-made bubble diagram of angiosperms. Special thanks are due to all whom kindly shared personal observations of orbicules with special mention of L. Ronse De Craene, F. Gonzalez and G. Prenner, and to all students at the Laboratory of Plant Systematics who contributed to the dense asterid and monocots sampling. S. Blackmore, M. Hesse, and the late J. Rowley are gratefully acknowledged by the last author for stimulating discussions on orbicules. A visit to RBG Edinburgh by the last author was funded by the European Commission's Research Infrastructure Action via the Synthesys Project (GB-TAF-5561). The Research Foundation - Flanders (FWO, G.0268.04, G.0250.05) and the KU Leuven (OT/05/35) financially supported our research. This work is dedicated to Gamal El-Ghazaly (1947–2001) and John Rowley (1926–2010).

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