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**[Review]**

# The Biology of Phenolic Containing Vesicles

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Phenolic compounds play a major role in the interaction of plants with their environment. They are thought to have been a feature of higher plants since early colonization of the land. Phenolics are crucial for many important aspects of plant life. They can play structural roles in different supporting or protective tissues, for example in cell walls, they can be involved in defence strategies, and signalling properties particularly in the interactions between plants and their environment. In brown algae, phenolic compounds are contained within membrane bound vesicles known as physodes, and their roles in algae are thought to be similar to those of higher plant phenolics. They can be stained using various histochemical stains, however, none of these stains are phenolic specific so care must be taken during interpretation of such results. Many, but not all phenolics are also autofluorescent under UV or violet light. Physodes are involved in cell wall construction, both in primary and secondary walls in brown algae. They bind together with other wall components to make a tough wall. They have also been found to play a role at fertilization, in blocking polyspermy in some species. Sperm are very quickly rendered immobile after phenolic release from newly fertilized zygotes seconds after fertilization. Phenolic compounds are thought to be important herbivore deterrents in some species due to their astringent nature. Phenolic compounds also offer effective UV protection in the early life stages and also the adults of many algal species. In the future, this factor may also make them an important player in the pharmaceutical and skincare industries.

**Key Words:** autofluorescence, cell walls, histochemistry, phenolics, physode, UV

## BROWN ALGAL PHYSODES

Physodes are membrane bound, spherical bodies containing phlorotannins. They stain black with osmium, blue with Toluidine Blue O, red with neutral red, orange with fast red GG and are autofluorescent under violet and UV excitation (Clayton and Ashburner 1994; Schoenwaelder and Clayton 1998a, b; Schoenwaelder 2002a).

Small light refracting bodies (probably physodes), were first described many years ago by Nägeli (1847) and Rosanoff (1868) (see Ragan 1976). Since then, many others have described them by many different terms including, brown algal bodies, fucosan granules and tannin bodies. It was Crato (1892) who first used the term physode, to describe the typically round to elliptical, mobile, vesicle-like, strongly light refracting bodies that he observed in cytoplasm of brown algae and other algae (Schoenwaelder 1996; Schoenwaelder 2002a).

Brown algal physodes are vesicles containing predominantly phenolic compounds, specifically phloroglucinol and derivatives of phloroglucinol (Ragan and Glombitza 1986). Brown algal polyphenolics, occurring in a single structural class-the phlorotannins, are thought to be synthesized via the acetate-malonate pathway (Targett and Arnold 1998). Their chemical structure has been investigated in many species of brown algae (Glombitza and Sattler 1973; Glombitza *et al.* 1973, 1975, 1977a, b, 1985, 1997; Glombitza and Rösener 1974; Ragan and Craigie 1976; Ragan and Jensen 1977; Gregson and Daly 1982; Ragan and Jamieson 1982; Ragan 1985; Blackman *et al.* 1988; Wölwer-Rieck and Glombitza 1990; Li and Glombitza 1991; Sailler and Glombitza 1999; Glombitza and Schmidt 1999). They are found in a range of molecular sizes, the high molecular weight phlorotannins most common (Glombitza and Rösener 1974; Ragan and Craigie 1976; Ragan and Jensen 1977, 1979).

Polyphenolic compounds are very difficult to isolate quantitatively, due to their large size, structural similarity and their reactivity with other compounds (Targett and Arnold 1998). Consequently, it is mainly indirect methods using colorimetric and microscopic techniques that have been used to measure phenolic concentrations

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in brown algae (Targett and Arnold 1998). However, there is great variation in the way the phenolics are extracted and the way in which they are characterized, causing difficulties when inter-study comparisons are attempted (Targett and Arnold 1998). Ways in which they can be qualitatively analysed include: chromatography (Craigie and McLachlan 1964; Ragan and Craigie 1978), high performance liquid chromatography (HPLC) (Wölwer-Rieck and Glombitza 1990; Li and Glombitza 1991; Glombitza and Schmidt 1999; Sailler and Glombitza 1999), nuclear magnetic resonance (NMR) spectroscopy (Glombitza *et al.* 1977a, b, 1985; Gregson and Daly 1982;Wölwer-Rieck and Glombitza 1990; Glombitza *et al.* 1997; Glombitza and Schmidt 1999; Sailler and Glombitza 1999), and other spectroscopy techniques (Glombitza *et al.* 1977b; Ragan 1985; Glombitza and Schmidt 1999).

**How do we look at phenolic vesicles?**

Studies on the tissue localization of phenolic compounds provide a fundamental prerequisite for understanding the ecological and physiological functions of these compounds. Without knowing where they are located within the cell and in the algae / plant as a whole, makes assigning role(s) difficult. Microscopy (both at the light and electron microscope level) is the only way to examine physodes both in living and fixed tissue and their relationship with other cellular constituents.

**Histochemistry of phenolics**

It is thought that histochemical investigation into physodes began around 1876 when Reinke (1876) stained brown algal cells with iron salts, iodine and osmic acid and tested their solubility in organic solvents (Schoenwaelder 2002a). Reinke (1876) concluded however, that as a result of his testing that these light-refracting bodies contained fats, a view which many of his contemporaries agreed with (Bruns 1894; Henckel 1902; LeTouzé 1912). However, as these stains are not specific to fats Ragan (1976) questioned the evidence leading to these conclusions.

Other early chemical studies have described physodes as being composed of a variety of other substances including proteinaceous material (Berthold 1882; Crato 1893a), carbohydrates (Hansteen 1892, 1900), terpene compounds (Ando 1951), nitrogenous compounds (Crato 1896) and glycosides (Hunger 1902; Ando 1951). Similarly, these conclusions were primarily based on the incorrect interpretation of the staining reactions produced by various histochemical stains (Schoenwaelder 2002a).

In 1886, Berthold suggested that physodes contained tannins, and this was confirmed (Hunger 1902; Kylin 1912; LeTouze 1912). Crato (1892, 1893a, b) was the first to suggest that physodes specifically contained phloroglucinol, a 1,3,5-trihydroxybenzenoid compound or a phloroglucinol derivative. Many early workers generally agreed with this assessment (Kylin 1918, 1938; Mangenot 1921; Dangeard 1930; Chadefaud 1932, 1934, 1936). The view that physodes contain phloroglucinol is widely accepted today (Glombitza and Sattler 1973; Glombitza *et al.* 1973; Ragan and Craigie 1976; Gregson and Daly 1982; Ragan and Jamieson 1982; Ragan 1985; Ragan and Glombitza 1986; Li and Glombitza 1991). The generally accepted definition of physodes is similar to that of Kylin's (1938) description of fucosan vesicles: "subcellular bodies in brown algal cells, whose contents colour red with vanillin-HCL, darken with $OsO_4$ (although this reagent may or may not fix them), are reducing, and are vitally stained with methylene blue, neutral red and cresyl blue" (Schoenwaelder 2002a).

Physodes will stain with many histochemical stains such as osmium tetroxide (Fritsch 1945; Chafe and Durzan 1973; Pellegrini 1980; Clayton 1992; Schoenwaelder and Clayton 1998a, b; Schoenwaelder and Clayton 1999a, b., 2000; Schoenwaelder 2002a, b; Schoenwaelder *et al.* 2003), neutral red (Chadefaud 1934; Fritsch 1945; Pellegrini 1980; Schoenwaelder 1996; Schoenwaelder and Clayton 1998a), toluidine blue O (McCully 1966, 1968; Evans and Holligan 1972; Leppard 1973; Brawley *et al.* 1976; Clayton and Ashburner 1994; Schoenwaelder 1996; Schoenwaelder and Clayton 1998a), fast red GG (Clayton and Ashburner 1994; Schoenwaelder 1996; Schoenwaelder and Clayton 1998a), vanillin HCL (Fritsch 1945) and cresyl blue (Mangenot 1921; Fritsch 1945; Pellegrini 1980). Some physodes also exhibit autofluorescence under violet and ultraviolet excitation (Clayton 1992; Schoenwaelder 1996; Schoenwaelder and Clayton 1998b; Schoenwaelder 2002a).

The existence of several biochemical classes of phenolic compounds or varying concentrations of a single compound may possibly cause differences in appearance in transmission electron micrographs (McCully 1966; Pellegrini 1980) and the variation of staining reactions with toluidine blue from green through turquoise to dark blue (McCully 1966; Evans and Holligan 1972; La Claire and West 1978; Schoenwaelder 1996; Schoenwaelder and Clayton 2000). The colour differences seen with tolu-

idine blue staining also appear to correspond to size class differences. Larger physodes appear turquoise and smaller physodes dark blue (Schoenwaelder 1996; Schoenwaelder and Clayton 2000).

Two types of osmiophilic vesicles are to be associated with Golgi bodies, the appearance of which depends on the method of fixation (Schoenwaelder and Clayton 2000). Chemically fixed preparations contain vesicles with osmiophilic particles as well as uniformly osmiophilic physodes; whilst those freeze substituted are uniformly osmiophilic. Such differences in appearance may correspond to the differences found with toluidine blue staining. It was proposed that the differences may reflect a lack of stability of newly synthesized phenolics, which are particularly susceptible to chemical fixation (and therefore appear patchy), before their deposition into fully osmiophilic physodes. It may explain why there has been no general agreement on the site of physode biogenesis in the past (Schoenwaelder and Clayton 2000).

In both higher plants and algae, investigation of non-coloured and non-fluorescent phenolic compounds has been carried out using techniques such as: immunocytochemical detection (Grandmaison and Ibrahim 1996), histochemical staining (Treutter 1989; Schoenwaelder and Clayton 1998a, b) or induction of secondary fluorescence e.g. flavonoid staining with Naturstoffreagenz A (Schnitzler *et al*. 1996; Reinold and Hahlbrock 1997; Hutzler *et al.* 1998).

**Staining and fixation problems**

At an ultrastructural level, the descriptions of differences in physode appearance may be due to inadequate fixation procedures (Schoenwaelder 2002a). In poorly fixed material the appearance of internal differentiation, bubbles and whorls within physodes is common (Hansteen 1892; Crato 1896; Chadefaud 1934; Clayton and Beakes 1983). Physodes have been described as fusing together to form large aggregates filling large portions of cells (Kaur and Vijayaraghavan 1992), but this is a common indication of poorly fixed material (Schoenwaelder 2002a). The multitude of different fixation procedures used over the years, tend to complicate comparisons between studies. Generally, any ultrastructural studies of phenolic-containing cells are difficult, primarily due to the reactivity of the physodes causing leaching into surrounding cytoplasm (Mueller and Greenwood 1978). Unless specialized fixation procedures are followed, (for example, with caffeine added to the fixative to stabilize the phenolics) it is very difficult to make reliable observations on physode structure and abundance in brown algal cells (Clayton and Beakes 1983). In all of the early studies in which different physode classes were identified based on appearance after fixation, no special precautions were taken to preserve physodes (McCully 1968; Evans and Holligan 1972; Pellegrini 1980; Kaur and Vijayaraghavan 1992) and hence may have resulted in artefacts (Schoenwaelder 2002a). Bouck (1965) compares the electron opaque phenolic material seen in electron micrographs of Fucus to the tannins seen in higher plants micrographs. High pressure freeze substitution (e.g. Xu *et al.* 2008) will almost certainly help in ascertaining more about physodes.

**Autofluorescence**

Fluorescence represents emission of visible light by organic molecules that had been excited by the absorption of UV radiation or short wavelength visible light (Buschmann and Lichtenthaler 1998). Many phenolic compounds in many different algae and plants are autofluorescent under violet or UV excitation. When excited by UV radiation, plants exhibit a fluorescence emission spectrum showing strong emission in the blue green region (400-550 nm) (Buschmann and Lichtenthaler 1998).

Brown algal physodes often exhibit autofluorescence under violet and UV excitation (Clayton 1992; Schoenwaelder and Clayton 1998a, b, 1999a, b.; Schoenwaelder 2002a, b). The phenolics in living eggs, zygotes, and embryos of many brown algal species autofluoresce in the green range when exposed to violet excitation and in the blue range when exposed to UV excitation (Schoenwaelder and Clayton 1998b).

In the adult thallus tissue of *Hormosira banksii*, physodes autofluoresce blue or blue-green, indicating the presence of phenolic compounds (Schoenwaelder and Clayton 1998b). Fluorescence microscopy is a powerful tool for studying tissue localisation of phenolics without chemically treating the cells. Their autofluorescence provides a good marker for physodes in living tissue where physodes predominantly accumulate in the peripheral cells of the thallus (Schoenwaelder and Clayton 1998b; Schoenwaelder 2002b).

A similar autofluorescence signal is also seen from higher plant phenolics, which also exhibit a characteristic UV induced blue-green autofluorescence (Harris and Hartley 1976; Parker and Waldron 1995; Lichtenthaler and Miehé 1997). Ferulic acid has been implicated in

cross-linking cell wall polysaccharides (Brett and Waldron 1996) and has been shown to exhibit a pH dependent autofluorescence (Harris and Hartley 1976; Parker and Waldron 1995; Hutzler *et al.* 1998; Opitz *et al.* 2003). Several classes of phenolics are strongly autofluorescent when irradiated by blue or UV light e.g. hydroxycinnamic acids, coumarins, stilbenes, styrylpyrones, and lignins (Hutzler *et al.* 1998; Donaldson 2001; Opitz *et al.* 2003; Combrinck *et al.* 2007). This blue-green fluorescence is mainly emitted from the epidermis, cell walls and veins, is an indicator of ferulic acids covalently bound to cell wall carbohydrates, and soluble cinnamic acids and flavonoids (Buschmann and Lichtenthaler 1998; Hutzler *et al.* 1998; Lichtenthaler and Schweiger 1998; Opitz *et al.* 2003; Combrinck *et al.* 2007).

It has also been reported that the blue-green fluorescence appears diminished in regions where there is more chlorophyll, this is thought to be due to re-absorption of blue-green fluorescence by photosynthetic pigments, which have a broad absorption band in the blue-green region (Lichtenthaler and Miehé 1997; Buschmann and Lichtenthaler 1998). This autofluorescent signature has been commonly used as an indicator of stress in higher plants (Schweiger *et al.* 1996; Lichtenthaler and Schweiger 1998).

This is not only seen in brown algae and higher plants, and mature zygospores of *Chylamydomonas monoica* exhibit a UV-induced blue surface autofluorescence indicating the presence of phenolic wall components (Daniel *et al.* 2007). In a wall defective mutant the autofluorescence was markedly reduced. It was suggested that the autofluorescence was derived from sporopollenin or from other UV- absorbing phenolic compounds loosely associated with or covalently linked to structural wall polymers (Daniel 2007).

Confocal Laser Scanning Microscopy (CLSM) may provide information on the subcellular localization of phenolic compounds, in particular whether or not the signal is derived from the vacuole or cell wall (Hutzler *et al.* 1998). This can be done without chemical intervention in many cases. Another advantage of CLSM may be the ability to obtain quantitative information about the fluorescence intensities (Hutzler *et al.* 1998). With the increased availability of UV lasers in many laboratories we may see more studies making use of these properties with phenolics.

**Physode movement around the cell.**

Physode movement has been observed by several researchers (Crato 1896; Hansteen 1900; Mangenot 1921; Chadefaud 1929; Evans and Holligan 1972). Hunger (1902) described physodes as oscillating in one position, whilst others describe an amoeboid or Brownian movement (Mangenot 1921; Chadefaud 1929). In the past, this physode movement was attributed to their semi-liquid or liquid composition (Crato 1896; Dangeard 1930; Defer 1930; Kylin 1938; Pellegrini 1980).

Today, actin is widely considered responsible for vesicle transport (Mollenhauer and Morre 1976; Url *et al.* 1993; Hable and Kropf 1998; Schoenwaelder and Clayton 1999a). In brown algae, inhibitors such as cytochalasin prevent polarization in zygotes by blocking actin-mediated transport of vesicles from perinuclear Golgi bodies to the rhizoid tip (Brawley and Quatrano 1979). They are also thought to disrupt vesicle target sites in the presumptive rhizoid, and thus prevent the localization and secretion of wall components such as fucoidan into the cell wall (Brawley and Robinson 1985; Quatrano and Shaw 1997).

Physodes accumulate at the rhizoid tip during polarization in healthy, untreated *Hormosira zygotes* (Schoenwaelder 1996; Schoenwaelder and Clayton 1998b). No accumulation is seen when the zygotes are treated with cytochalasin and latrunculin, suggesting that actin is responsible for physode movement to the rhizoid tip. Germination in fucoids is also affected by actin inhibitors (Nelson and Jaffe 1973; Quatrano 1973; Brawley and Quatrano 1979; Brawley and Robinson 1985; Schoenwaelder and Clayton 1999a).

Studies on algae and plants using cytochalasin treatment commonly show secretory vesicles accumulating around Golgi bodies in the perinuclear region, and not moving to other parts of the cell (Mollenhauer and Morre 1976; Brawley and Quatrano 1979; Pope *et al.* 1979; Ridge 1990; Williamson 1993; Schoenwaelder and Clayton 1999a). As well as accumulation of vesicles in the perinuclear region, there is a depletion of vesicles near the rhizoid tip. These observations are consistent with cytochalasin stopping vesicle transport to the rhizoid tip without inhibiting secretion of vesicles already there (Brawley and Quatrano 1979; Pope *et al.* 1979; Williamson 1993).

Although microtubule inhibitors do not prevent polarization and rhizoid formation in fucoids (Kropf *et al.* 1990; Kropf 1992; Schoenwaelder and Clayton 1999a), they do prevent physodes accumulating in the rhizoid tip during polarization (Schoenwaelder and Clayton 1999a). It was proposed that actin transports physodes

away from the site of synthesis in the perinuclear region, whereas functional microtubules are necessary for transport to the rhizoid tip (Schoenwaelder and Clayton 1999a).

In studies on higher plants, microtubule inhibitors can result in the redistribution of Golgi bodies to peripheral sites throughout the cell (Kreis 1990; Cole and Lippincott-Schwartz 1995). It is thought that the Golgi remains functional but is unable to direct material to the plasma membrane (Ridge 1990). This study suggested that the F-actin system acts as a general delivery and circulatory mechanism, with the microtubules directing the vesicles in the root hair tip to the site of wall deposition. In line with this, Schoenwaelder and Clayton (1999a, b) proposed that this might be occurring in fucoids. Physode movement to regions of active wall formation (starting from where they are formed, the perinuclear region, to the cell periphery, the growing rhizoid tip and the impending plane of cytokinesis during brown algal development) is dependent on the cytoskeleton (Schoenwaelder and Clayton 1999a). Both microfilaments and microtubules are involved, but they appear to play differing roles in physode movement at different times during development. Thus the actin cytoskeleton acts as a general system for moving physodes away from where they are produced to regions where they are required. Microtubules, in conjunction with the microfilaments, then control the movement to specific accumulation and deposition sites in the rhizoid tip and the impending plane of cytokinesis (Schoenwaelder and Clayton 1999a).

**How large are physodes?**

Physodes vary in size from 0.1-10 $\mu$m in diameter (Kylin 1918; Fritsch 1945; Evans and Holligan 1972; Schoenwaelder 1996; Schoenwaelder and Clayton 1998a, b), with an average size of 1-4 $\mu$m reported in the literature (Ragan and Glombitza 1986). They are thought to be bound by a limiting membrane, composed either of a typical membrane (Bisalputra *et al.* 1971; Loiseaux 1973; Pellegrini 1980; Schoenwaelder 2002a) or alternatively, one of unknown structure (Evans and Holligan 1972).

Historically, various physode classes have been described by different authors (Le Touze 1912; Kylin 1918; Chadefaud 1934; Berkaloff 1962; Feldmann and Guglielmi 1972; Brawley *et al*. 1976; Ragan 1976; Pellegrini 1980; Ragan and Glombitza 1986; Schoenwaelder and Clayton 2000), with some studies attributing differences in physode appearance to different stages in their development (Berkaloff 1962; Evans and Holligan 1972; Pellegrini 1980; Schoenwaelder and Clayton 2000). The significance of the differences seen in physode appearance is not fully understood.

**Where are they are located in the cell?**

Spores, gametes and zygotes of brown algae commonly contain physodes (Clayton 1992; Schoenwaelder and Clayton 1998a, b, 2000; Schoenwaelder and Wiencke 2000; Schoenwaelder 2002a). The abundance and location of physodes varies in different kinds of propagule and in different species. Physodes are also present in vegetative tissue (Schoenwaelder 2002b).

At the time of release from the conceptacle, fucoid eggs contain large concentrations of physodes (Schoenwaelder 1998a, b; Schoenwaelder and Wiencke 2000). In eggs and young zygotes, physodes accumulate around the cell periphery and are also sometimes found in large numbers in the perinuclear region (Schoenwaelder and Clayton 1998a, b; Schoenwaelder and Clayton 1999a, b; Schoenwaelder and Clayton 2000; Schoenwaelder and Wiencke 2000; Schoenwaelder 2002a). Accumulation of peripheral physodes, or what has been described as "other unidentified vesicles" (probably physodes), have also been noted in many studies (e.g., Thuret 1858; Farmer and Williams 1896, 1898; Le Touzé 1912; Levring 1947; Bouck 1965; Brawley *et al.* 1976; Takamura; 1976). Accumulation around the nucleus is also very common (Crato 1892; Le Touzé 1912; Richard 1929; Fritsch 1945; Evans *et al.* 1982; Phillips *et al.* 1994; Schoenwaelder and Clayton 1999a, b, 2000). This can be simply explained due to the fact that physodes are thought to be produced in the perinuclear region – in the endoplasmic reticulum and the Golgi (Schoenwaelder and Clayton 2000).

Physodes are found during germination, accumulating in the rhizoid tips of fucoids (Schoenwaelder 1996; Schoenwaelder and Clayton 1998b; Schoenwaelder and Wiencke 2000), and in the plane of the impending cross-wall, just prior to cell division (Schoenwaelder 1996; Schoenwaelder and Clayton 1998b; Schoenwaelder and Wiencke 2000). Physodes also accumulate along the future plane of cytokinesis of the sporangium in some members of the Dictyotales (Phillips *et al.* 1994. They are incorporated into the cell walls of many species (Schoenwaelder and Clayton 1998a, b, 1999b; Schoenwaelder and Wiencke 2000; Schoenwaelder 2002a).

**Where are physodes produced?**

Physodes are probably produced in the perinuclear

region, by ER and Golgi bodies (Schoenwaelder and Clayton 1999a, 2000; Schoenwaelder 2002a). Cytoskeletal inhibitor studies have shown that physodes are produced in the perinuclear region, in vesicles derived from the ER and Golgi bodies (Schoenwaelder and Clayton 2000). ER cisternae in *Phyllospora comosa* had osmiophilic bud-like outgrowths, which were closely associated with larger osmiophilic vesicles (physodes). In higher plants, phenolic-containing vesicles are also thought to arise from the ER (Chafe and Durzan 1973; Baur and Walkinshaw 1974; Parham and Kaustinen 1977; Zaprometov *et al.* 1994).

Historically, there has been much controversy as to where phenolic compounds are synthesized and packaged within the cell. Many locations have been suggested including, the cell cytoplasm (Crato 1892; Chadefaud 1927; Dangeard 1930; Kylin 1938), cell vacuoles (Mangenot 1922; Berkaloff 1962), chloroplasts (Hansteen 1892, 1900; Hansen 1895; Henckel 1902; Hunger 1902; Kylin 1912, 1918; Evans and Holligan 1972; Pellegrini 1980; Kaur and Vijayaraghavan 1992), the chloroplast membrane (Kylin 1918; McCully 1968; Davies *et al.* 1973), chloroplast endoplasmic reticulum (Bouck 1965; Feldmann and Guglielmi 1972; Oliviera and Bisalputra 1973; Ragan 1976), the endoplasmic reticulum (Mangenot 1921; Defer 1930; Feldmann and Guglielmi 1972; Davies *et al*. 1973; Ragan 1976; Schoenwaelder and Clayton 2000), Golgi bodies (McCully 1968; Schoenwaelder and Clayton 2000).

In both brown algae and in higher plants phenolic containing vesicles grow in size by fusion. In higher plant cells, tannin vacuoles are thought to be formed by the fusion of small vacuoles (Parham and Kaustinen 1977) and some researchers have suggested that physodes in brown algae may become larger in the same way. There are many accounts of phenolic containing bodies fusing to form larger vesicles (Hansteen 1900; Kylin 1918; Fritsch 1945; Bisalputra *et al.* 1971; Evans and Holligan 1972; Pellegrini 1979, 1980). There have also been reports of physodes dividing and multiplying, spherical outgrowths have been described that are involved in a budding process (Chadefaud 1929, 1934; Fritsch 1945; Schoenwaelder 1996; Schoenwaelder and Clayton 1998b; Schoenwaelder 2002a).

## WHAT TO PHYSODES ACTUALLY DO?

### Primary cell walls

Phenolics are an important component of higher plant and fucoid cell walls (Fry 1979, 1982; Harris and Hartley 1980; Biggs and Fry 1987; Bolwell 1993; Wallace and Fry 1994; Parker and Waldron 1995; Schnitzler *et al.* 1996; Schoenwaelder and Clayton 1998b, 1999a, b; Wojtaszek 2000). In higher plants in they are most often bound to matrix polymers (Fry 1983). The phenolics are predominantly deposited in the secondary cell walls, where they are implicated in thickening, but they are also thought to be biologically active constituents of the primary wall (Fry 1986; Wallace and Fry 1994) where, under the control of peroxidases, they cross-link with carbohydrates (Fry 1983, 1989; Biggs and Fry 1987).

The main components of brown algal cell walls are alginate, alginic acid, fucoidin, cellulose (Brawley *et al.* 1976; Quatrano and Stevens 1976), and phenolic compounds (Schoenwaelder 1996; Schoenwaelder and Clayton 1998b, 1999a, b; Schoenwaelder and Wiencke 2000). The walls consist of fibrillar material embedded in an amorphous matrix (Dawes *et al.* 1961; McCully 1968; Mariani *et al.* 1985). In *Fucus*, alginates are secreted soon after fertilization of the egg. Other major wall constituents, cellulose and fucans, are detectable within 20 min and 1 h of fertilization, respectively (Brawley *et al.* 1976; Vreeland and Laetsch 1988). The relative proportions of the three major constituents are similar after 1 h (Quatrano and Stevens 1976), and, by 6-24 h after fertilization, the wall composition stabilizes with the proportions of alginic acid, cellulose, and fucans remaining constant at 3:1:1 (Quatrano and Stevens 1976; Vreeland and Laetsch 1988).

Physodes accumulate at the zygote periphery early in development and are secreted into the primary zygote wall (Schoenwaelder and Clayton 1998a, b, 1999b; Schoenwaelder and Wiencke 2000; Schoenwaelder 2002a). They are secreted in two ways, whole physodes undergo exocytosis, and their phenolic contents permeate the wall, or, small phenolic-containing particles budding off from physodes near the cell surface are secreted into the wall (Schoenwaelder and Clayton 1998b; Schoenwaelder 2002a).

In zygotes of *Hormosira* and *Acrocarpia* phenolic compounds are secreted into the wall from the time of fertilization (Schoenwaelder 1996; Schoenwaelder and Clayton 1998a, b). In *Acrocarpia*, cell wall formation begins with the release of phenolic bodies from the newly fertilized zygote. Subsequently, vesicles having a granular appearance (possibly containing peroxidases) in the TEM appear near the zygote membrane and undergo exocytosis, apparently causing a stabilization of the phe-

nolic layer (Schoenwaelder and Clayton 1998a). This stabilization may occur via cross-linking as proposed by Vreeland and Laetsch (1988). More recently, Salgado *et al.* (2007) confirmed that alginate links strongly with phenolic compounds, and that this phenomenon may also occur *in vivo*.

Once outside the cell membrane, phenolic bodies break up, probably as they are no longer membrane bound, and small particles of phenolic material become embedded in the zygote wall. A variety of histochemical staining reactions also indicate the presence of phenolic constituents in isolated zygote cell walls (Schoenwaelder and Clayton 1999b). The phenolic deposits are concentrated in the layers of the wall closest to the cell contents in intact cells. As cell wall construction is of such primary importance to cell architecture, it would seem reasonable that the involvement of phenolics in their construction would be generalized to at least all fucoids, if not all brown algae. There is good evidence that phenolic compounds are involved in the early development of fucoids, their role as herbivore deterrents may be a consequence of their role in wall construction (Schoenwaelder and Wiencke 2000; Schoenwaelder 2002a).

**Polarization**

In most fucoids, germination occurs with 24 h after fertilization (Farmer and Williams 1898; Novotny and Forman 1974; Quatrano 1978; Evans *et al.* 1982; Berger and Brownlee 1995; Quatrano and Shaw 1997; Schoenwaelder and Clayton 1998b; Schoenwaelder and Wiencke 2000). Physodes accumulate in the presumptive rhizoid tip and then in the developing rhizoids and can be seen using various histochemical stains as well as autofluorescence (Schoenwaelder and Clayton 1998a, b; Schoenwaelder and Wiencke 2000).

**Cross Walls**

During cross wall formation, phenolics appear as a strongly marked band stretching across the centre of the cell (Schoenwaelder and Clayton 1998b). Staining living zygotes with Calcofluor White and Toluidine Blue both failed to reveal the presence of wall polysaccharides prior to the formation of this physode band. Soon after the physodes line up, coalescing electron translucent vesicles also appear in the same plane, and, shortly thereafter, the membranes dividing the cell into two are apparent (Schoenwaelder 1996; Schoenwaelder and Clayton 1998b). This also occurs in Fucus sp. (Schoenwaelder and Wiencke 2000). The phenolics are the first components to arrive at the forming cross walls.

Few early studies used fixation techniques that resulted in good preservation of physodes, and without adequate fixation, their involvement in cross-wall formation may have been missed (Schoenwaelder 2002a). However, in 1896, Crato described physode involvement in cell division and suggested that the physodes were important in the formation of the cell wall because they accumulated in the forming cell plate region of Chaetopteris (now Sphacelaria) in much the same way as they do in Hormosira and Acrocarpia.

**Polyspermy Block/Adhesive**

Polyspermy in algae can lead to abnormalities, and ultimately embryo death or survival (Brawley 1987). A reliable mechanism for preventing this condition is essential for species. In *Fucus* and *Pelvetia* the change in membrane potential caused by sperm entry lasts for several minutes and functions as a fast block to polyspermy (Brawley 1987, 1990, 1991). A more permanent block is established with the secretion of cell wall materials, notably alginate and sulfated fucans (Stevens and Quatrano 1978; Brawley and Quatrano 1979; Evans *et al.* 1982; Brawley and Bell 1987; Brawley 1990). However, in many species there is a short time interval during which the newly fertilized zygote has no protection against polyspermy (Brawley and Bell 1987; Brawley 1990, 1991). Brawley (1990) postulated the existence of an intermediate polyspermy block to protect the zygote during this period, and Schoenwaelder (1996) suggested that phenolics might function in this manner.

Chadefaud (1932) was one of the first to describe the secretion of physodes from cells of *Myrionema vulgare*. Since then, there have been many reports of brown algae secreting phenolic compounds from vegetative tissue into surrounding seawater, causing a brown discolouration of the water (Schoenwaelder 1998a). Phenolics are also released by fertilized zygotes (Clayton and Ashburner 1994; Schoenwaelder and Clayton 1998a). The triggering of phenolic secretion in newly fertilized zygotes has been documented in *Durvillaea potatorum*, in which small peripheral phenolic vesicles were secreted within a few minutes of spermatozoid entry (Clayton and Ashburner 1994). This massive release is also seen in *Acrocarpia paniculata* but on a much larger scale (Schoenwaelder and Clayton 1998a). In much greater volumes, the amount of secreted phenolic material in *Acrocarpia paniculata* upon fertilization, causes a distortion of the cell membrane, visible by light microscopy

(Schoenwaelder and Clayton 1998a). The zygotes then adhered to the substratum (Schoenwaelder and Clayton 1998a). Schoenwaelder and Clayton (1998a) compared this massive exocytosis of phenolics in scale and timing to the release of cortical granules in sea urchin eggs. Coinciding with this massive release of phenolics the motile spermatozoids near the egg slow down and stop. The phenolic material is then incorporated into the primary wall of the newly fertilized zygotes, which appear as a yellowish / brownish oxidised layer (Schoenwaelder 1998a).

Just after fertilization in *F. serratus*, the movement of sperm surrounding fertilized zygotes is affected (Schoenwaelder and Wiencke 2000). Similar behaviour has been noted in other brown algae, including *Halidrys* (Farmer and Williams 1898), *Cytoseira barbata* (Knapp 1931), *Fucus vesiculosus* (Levring 1947, 1952) and *Acrocarpia paniculata* (Schoenwaelder and Clayton 1998 a). In *Acrocarpia*, phenolic compounds are secreted from the egg upon fertilization, and this is correlated with distortion of the cell membrane and slowing of sperm.

**Herbivore Defence**

Some studies have suggested a link between phenolic content and geographic location. Phenolic compounds occur in high concentrations in temperate brown algae in Australasia (Steinberg 1985; Estes and Steinberg 1988), but are lower in North American species belonging to the same order (Hay and Fenical 1988; Steinberg 1989; Hay and Steinberg 1992). However, contrary to this pattern, phenolic concentration does not seem to show any geographical relationship in tropical systems (Steinberg 1986; Steinberg and Paul 1990; van Alstyne and Paul 1990; Targett *et al.* 1992). The idea that phenolics act as a herbivore deterrent was first suggested by Hunger (1902) and is widely accepted (Tugwell and Branch 1989; Hay and Fenical 1992; Steinberg and van Altena 1992; van Altena and Steinberg 1992). The comparatively low phenolic concentrations found in North American brown algae deter a range of herbivores, but conflicting results show that Australasian invertebrate herbivores are unaffected by higher levels of phlorotannins (Steinberg and van Altena 1992). The lack of consistency in these studies suggests that phlorotannins play one or more alternative roles to herbivore defence in brown algae.

**UV Protection**

Phenolic compounds provide protection from ultraviolet (UV) radiation (Schoenwaelder 2002a, b; Schoenwaelder *et al.* 2003). In fact, one of the first functions attributed to physodes was that of protection against excess irradiance (Berthold 1882; McLachlan and Craigie 1964). Even a small increase in total UV-B may cause significant biological damage, with potential changes including DNA damage, changes to proteins and membranes, alterations in transpiration and photosynthesis and changes in growth, development and morphology (Jansen *et al.* 1998). In addition, increases in UV-A produce accumulations of flavonoids and or potential protective products in the vacuoles of the epidermis cells in higher plants (Buschmann and Lichtenthaler 1998). As polyphenolics are typically concentrated in the epidermal and meristematic cells of adult brown algae, and around the periphery of eggs, zygotes and embryos of early stages, they may protect cells from excess UV radiation in the same way (Schoenwaelder 2002a, b; Schoenwaelder *et al.* 2003).

In higher plants, UV-A and UV-B induces accumulation of a range of secondary metabolites including phenolic compounds. This was observed recently in *Ascophyllum nodosum* where extracts from this alga absorbed in the UV-B range of the spectrum (Pavia *et al.* 1997), and in *H. banksii* where physodes accumulated in the outer tissue layers (Schoenwaelder 2002b).

The autofluorescent phenolic compounds, contained within physodes, are present in the peripheral cells, cell walls, around conceptacles, in holdfasts and meristematic regions and may play a role in protection of the alga from UV radiation. (Schoenwaelder 2002b).

The protective effects of brown algal phenolics have further been investigated in algae and in mice. Liquid phloroglucinol has been used inside UV transparent Plexiglas filters to test its protective effects against UV radiation on early life stages of *Fucus serratus* (Schoenwaelder *et al.* 2003). When the usually UVR sensitive *F. serratus* zygotes were covered by filters containing 4% phloroglucinol, and exposed to different UVR treatments, normal development continued under all treatments. Whereas, with the treatments exposed to UVR, with no phenolic containing filter, the zygotes were detrimentally affected by both UVA and UVB radiation. Zygotes were unable to polarise, germinate and divide; they ultimately died (Schoenwaelder *et al.* 2003).

The protective effect of brown algal phenolics against UV radiation in mice was tested through both dietary and topical administration trials (Hwang *et al.* 2007).

These trials produced a marked inhibition of cell proliferation and cyclooxygenase-2 activity. This study sug-

gested that brown algal phenolics impart an anti-photocarcinogenic effect, which may be associated with the prevention of UVB-induced oxidative stress, inflammation and cell proliferation in the skin. Both the topical and ingested methods of administration gave similar results. The authors concluded that brown algal polyphenols were highly protective against UVB-induced skin carcinogenesis (Hwang *et al.* 2007). Brown algal phenolics, it seems, may be promising compounds in terms of protection against UV radiation, particularly in the pharmaceutical and skin care industries.

## CONCLUSIONS

Phenolics are a complex class of compounds. Contained within membrane bound physodes they are found in all life stages of brown algae. They can be detected with various microscopy methods including traditional histochemistry and autofluoresce, and at the light and electron microscopy levels. Historically, the highly reactive nature of phenolic compounds has caused major problems during their microscopic investigation. They are often located both at the periphery of cells, as well as in perinuclear regions, where they are thought to be produced. Physodes are moved around the cell via the cytoskeleton, as both microfilaments and microtubules have been implicated in their movement.

The ubiquitous presence of phenolic compounds in the Phaeophyceae is a strong indication of their significance, and indeed, this presence suggests that they have been one of the fundamental features responsible for the evolutionary success of the division. They play a multiplicity of roles within the life cycle of the algae, from the early developmental stages, (such as eggs, zygotes) to the adult plants in areas such as: cell wall formation, adhesion, polyspermy prevention, defence and UV protection.

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