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Induction of phlorotannins through mechanical wounding and radiation conditions in the brown macroalga *Laminaria hyperborea*

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The accumulation of phlorotannins after mechanical wounding and exposure to ultraviolet (UV) radiation was investigated in *Laminaria hyperborea*. Grazer action was simulated by mechanical wounding of the thalli with a cork borer, and investigated 0, 1, 2, 3, 5, 7 and 9 days after wounding. Simultaneously, the effects of exposure to photosynthetically active radiation (PAR) (25–30 µmol photons $m^{-2} s^{-1}$) and additional UV radiation (7.5–8.8 $W m^{-2}$ UV-A; 0.45–0.5 $W m^{-2}$ UV-B) on the distribution of phlorotannins and structural changes were examined by light and fluorescence microscopy. Most phlorotannin-containing physodes were found in the outer cortex cells. However, some physodes were also present in the epidermis and in the medulla. Structural wound healing is divided into four stages: (1) gluing of the wound, (2) closing of the medullary cells to stop 'bleeding', (3) structural sealing of the medulla and enrichment of physodes in medullary cells and (4) cell divisions and re-differentiation of the cortex and medullary cells. The results suggest phlorotannins play a role in both wound sealing and reconstruction during wound healing. UV radiation has no effect on wound healing, but under both PAR and PAR with additional UV radiation an increase in the number of physodes is observed in the outer cortex and epidermal cells, indicating a reaction to high light stress. Overall, we show that phlorotannins play an important role during wound healing, and are inducible by PAR and PAR + UV radiation, indicating a function as photo-protective compounds.

Key words: brown algae, *Laminaria hyperborea*, PAR, phlorotannin induction, UV radiation, wound healing

Introduction

Phlorotannins are multifunctional secondary metabolites of marine brown algae (Phaeophyceae), which occur in a wide range of molecular sizes. Located in vesicles in the cytoplasm (physodes), phlorotannins are also a constituent of cell walls. These compounds can make up to 20% of the dry weight (Ragan & Glombitza, 1986). The size of physodes varies from 0.1 to 10 µm in diameter (Fritsch, 1959; Döpfner *et al.*, 1990; Schoenwaelder & Clayton, 1998*a*), with a mean size between 1 and 4 µm in diameter (Ragan & Glombitza, 1986). The occurrence and role of physodes in brown algae were reviewed by Schoenwaelder (2002*a*). Several functions of phlorotannins have been proposed. Phlorotannins are integral structural components of cell walls (Schoenwaelder & Clayton, 1998*b*), and in fucalean species are involved in the process of adhesion and settlement of newly fertilized zygotes. They accumulate at the zygote periphery and are then secreted into the primary zygote wall (Schoenwaelder & Clayton, 1998*a*, 1998*b*, 1999). Phlorotannins are able to bind bivalent ions (Döpfner *et al.*, 1990). This capacity has been inferred to bind most of the intracellular heavy metals, thereby keeping the cytoplasm free from toxic ions. This enables the algae to live in potentially polluted habitats (Ragan & Glombitza, 1986; Schoenwaelder, 2002*a*).

Another important and often discussed function of phlorotannins is protection against UV radiation. Since the middle of the 1980s, overall ozone levels in the stratosphere have decreased, with high regional and seasonal variations. The decrease in ozone results in a shift of the solar spectrum in the direction of shorter UV-B (280–320 nm) wavelengths (Franklin & Forster, 1997), resulting in damage to biologically important molecules and structures, in particular DNA, membrane lipids and the photosynthetic apparatus (Franklin & Forster, 1997; Karsten *et al.*, 2009). There are,

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however, mechanisms by which damage can be repaired and protective mechanisms that prevent damage before it occurs. In this respect, phlorotannins are hypothesized to have a protective function in brown algae (Pavia *et al.*, 1997; Pavia & Brock, 2000; Schoenwaelder, 2002*a*, 2002*b*), including spores (Wiencke *et al.*, 2000, 2004; Roleda *et al.*, 2006*a*, 2006*b*).

Beside their other functions, phlorotannins are commonly considered to act as chemical defences against herbivory, although experimental evidence to date remains inconclusive (e.g. Targett & Arnold, 1998; Amsler & Fairhead, 2006). Herbivore deterrence may be linked to specific properties of phlorotannins in relation to grazers (Amsler *et al.*, 2009). Two strategies utilized by algae can be distinguished. If a constant level of phlorotannins is maintained in the tissue, it is termed 'constitutive' defence. When formation of phlorotannins is stimulated, e.g. by herbivore attack, it is 'inducible'.

In *Laminaria hyperborea* the phlorotannin content is higher in older parts of the blades than in the meristematic regions, suggesting greater herbivore deterrence in these parts, while in the younger parts energy is mainly used to build new biomass (Connan *et al.*, 2006). Toth & Pavia (2002) did not show any phlorotannin induction in response to natural herbivory when measuring the entire phlorotannin content and concluded that in *L. hyperborea* phlorotannins are not inducible.

In a microscopical study, Lüder & Clayton (2004) showed induction of phlorotannins in response to simulated herbivory in *Ecklonia radiata*. In this species structural wound healing is divided into three different stages: closing of medulla, accumulation of phlorotannins in the wound area, and formation of a new epidermis. The study demonstrated for the first time that phlorotannins are inducible in *E. radiata* (Lüder & Clayton, 2004).

In the present study we investigate wound healing of *L. hyperborea* with different methods of microscopy. The occurrence and possible functions of phlorotannins in different stages of wound healing are studied. Additionally, the effect of UV radiation on wound healing and the distribution on phlorotannin-containing physodes within the thallus are tested.

Materials and methods

Algal material and experimental design

In September 2005, 2- to 3-year old sporophytes of *Laminaria hyperborea* were collected from a depth of 10 m at Helgoland (North Sea, 54°10 57′ N, 7°53 07′ E). The blade 'fingers' of six individuals were cleaned with paper tissues and then acclimated for 2 days to the conditions in the culture room, at 16:8 h light:dark cycles, 30 µmol photons $m^{-2} s^{-1}$ of white light, 10 ± 1°C in filtered sea water (2 µm filter; Millipore) without any added nutrients, and aerated rigorously with filtered air.

For the experiment, blade fingers with a width of 4–6 cm were cut into 4–5 cm long segments ($n = 16$). Segments of each individual were placed into 600 ml crystallizing dishes with filtered sea water. In each dish four segments were placed in such a way that they lay flat without shading each other. Mechanical wounding was simulated by punching one hole in the middle of each segment with a cork borer (5 mm in diameter). Two segments of each individual were left uninjured to act as controls. To ensure some water movement the dishes were placed on shaker tables (GfL 3013, Gesellschaft für Labortechnik, Burgwedel, Germany) in a 10°C culture room. After 4 days the medium was changed.

Structural changes around the hole were studied in freshly wounded segments (0 d control) and after 1, 2, 3, 5, 7 and 9 days. On day 9, the uninjured segments were freshly injured (9 d control). At each sampling time, two segments of each individual were removed, one for *in vivo* fluorescence microscopy, the other one for chemical fixation. For examination of the effect of different radiation conditions, all samples were placed under two fluorescence tubes emitting PAR (True Light® II Powertwist, 36 W Preheat, USA) and three UV radiation fluorescence tubes (Q-Panel UV A-340, 40 W, Cleveland, USA). Different cut-off filters were used to generate the two radiation conditions, with three individuals per radiation condition. At the same time, the foil prevented evaporation of the medium. For the PAR samples, radiation ≤ 400 nm was blocked (400 nm Folie Ultraphan URUV, 120 µm gauge, DIGEFRA Folienvertrieb, München). For the PAR + UV samples, radiation ≤ 295 nm was excluded (295 nm Folie, Ultraphan URT300; 0.3 µm thickness, DIGEFRA Folienvertrieb, München, Germany). Quartz glass dishes were used for the latter samples.

Experimental conditions were as follows: PAR was 25–30 µmol photons $m^{-2} s^{-1}$, measured with a Li-Cor Quantum Radiometer (Li-189, Li-Cor, Lincoln, USA), equipped with a flat head sensor (Li-Cor Quantum Q 31775, Li-Cor), UV-A radiation was 7.5–8.8 W m^{-2} and UV-B radiation was 0.45–0.5 W m^{-2}, measured with a Solarlight Quantum Radiometer (Solarlight, Philadelphia, USA), equipped with UV-A und UV-B flat head sensor (UV A- Detector PMA2116 und UV B- Detector PMA2106, Solarlight).

Light and fluorescence microscopy of living material

Living tissue was sectioned by hand with a razor blade parallel to the longitudinal axis of the blade as described in Lüder & Clayton (2004) and examined under bright field illumination. For auto-fluorescence a light microscope was used operating in epi-fluorescence mode (Axiophot photomicroscope, Zeiss, Oberkochen, Germany). Phlorotannins were imaged as green fluorescence using an excitation filter beamsplitter FT 510 nm and a barrier filter 525–565 nm (Zeiss®,

blue light). Phlorotannins were imaged following Lüder & Clayton (2004) as white to light blue-green fluorescence together with an orange fluorescence of chlorophyll using excitation filter BP 395-440 nm (violet-blue light), FT 460 nm and long bandpass barrier filter LP 470 nm.

Staining and light microscopy of fixed material

For fixation the specimens were cut into triangles as for fluorescence microscopy. The triangles were then fixed according to Schoenwaelder & Clayton (1998*a*). Immediately after cutting, triangles were transferred into 2% (w/v) glutaraldehyde, 1% (w/v) paraformaldehyde, 1% (w/v) caffeine, 2% (w/v) sodium chloride and 0.1% (w/v) CaCl in cacodylate buffer, pH 7.2, and fixed for 5 h at room temperature. Two triangles were then post-fixed in 1% (w/v) osmium tetroxide in cacodylate buffer for 4 h. All specimens were dehydrated through an ethanol series. The aldehyde-only samples were infiltrated with medium grade LR-White, and the aldehyde-osmium samples with Spurr's resin. After infiltration, specimens were polymerized at 60°C in flat embedding moulds. Semi-thin sections (2 µm) of material embedded in LR White were either stained with toluidine blue O (pH 4.5; TB) as described by Feder & O'Brien (1968) or with fast red GG (0.1% W/v, pH 5.2; Clark, 1981). All thin sections were capped with Euparal (Roth®) and examined under a light microscope in bright field.

Toluidine blue O stains polyphenols and lignin turquoise-green to blue-green, carboxylates and sulphidic polysaccharides, polyphosphates, polysulphates and polycarbonates violet, and peptide acids red to reddish. DNA is stained blue to blue-greenish, while cellulose is not stained at all (O'Brien & McCully, 1981). Fast red GG stains specific polyphenols and aromatic amines reddish orange (Clayton & Ashburner, 1994), and osmium stains phlorotannins intense black (Lüder & Clayton, 2004).

Statistics

To quantify the increase in physodes we used the Whitney–Mann U-test. Physodes are globular structures all similar in size. The amount per cell in epidermis and outer cortex cells was taken as a value with which to compare the 0 d control and the 9 d treatment and 9 d control for all individuals under PAR and PAR + UV conditions ($n = 3 + 3$).

Results

Distribution of phlorotannins in L. hyperborea *blades*

The cross-section of a blade of *L. hyperborea* shows three different kinds of tissue (Fig. 1*a*). At the blade surface there is a layer of epidermal cells, followed by the cortex and the medulla in the middle. In the epidermal cells 3 to 4 chloroplasts

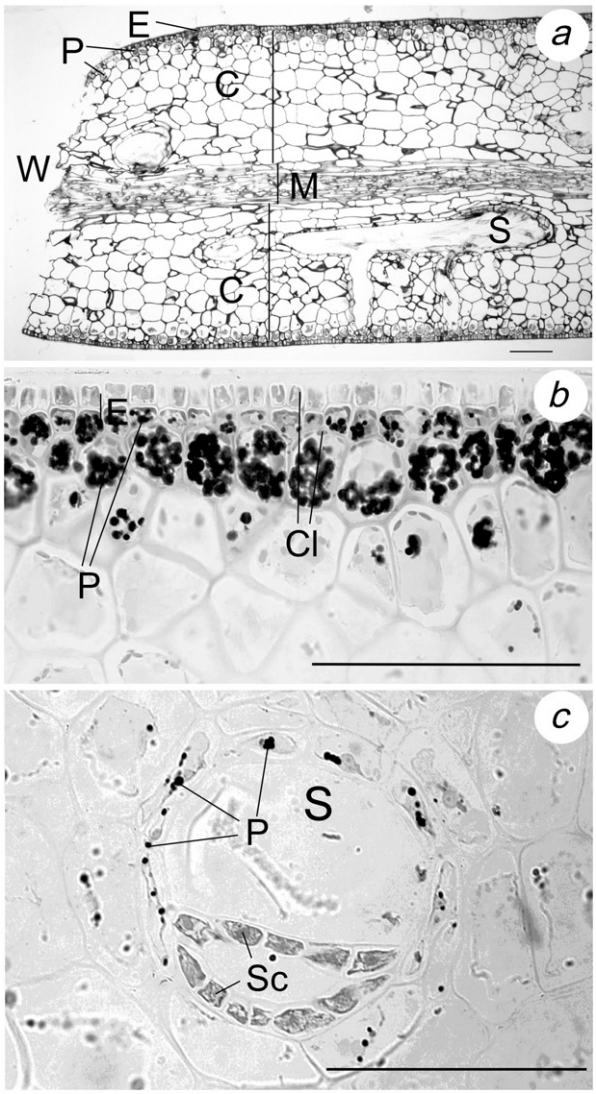

Fig. 1. Distribution of physodes in a freshly wounded blade (0 d control); (*a*) overview of a freshly wounded blade (toluidine blue O); (*b*) detailed view of epidermis and outer cortex (Os); (*c*) detailed view of a slime channel with secretion cells (Os). Abbreviations: C: cortex, Cl: chloroplast, E: epidermis, M: medulla, P: physodes, S: slime channel, Sc: secretion cells, W: wound site. Scale bars represent 100 µm.

are clearly visible in each cell (Fig. 1*b*). Physodes are scarce in the epidermal layer.

The cortex consists of huge cells with large vacuoles. The majority of the physodes are found in the outer cortical layer and only sporadically in the cells of the inner cortex (Fig. 1*a*, *b*). Few chloroplasts are visible in the outermost cells of the cortex (Fig. 1*b*). The slime channel system in the cortex is conspicuous (Fig. 1*a*, *c*), composed of longitudinally orientated main channels, from which smaller channels branch off and end at the epidermis. Slime-forming secretion cells occur at the broadenings of the channels (Fig. 1*a*, *c*). The channels are lined with a cell layer which contains a considerable number of physodes (Fig. 1*c*). In the medulla, trumpet cells with characteristic sieve plates are arranged longitudinally through the blade (Fig. 1*a*).

Structural changes after wounding

Wound healing was monitored on fixed and stained material over a period of 9 days (Figs 2, 3). Two days after wounding no structural changes were apparent in either the medulla or in the cortex. After 3 days, the medullary cells contained phenolic substances, and the medulla at the wound surface became slightly denser and the matrix became darker in toluidine blue O compared with the medulla behind the wound area (Fig. 2*c*, *d*). After 7 days, the cortical and medullary cells divided by forming new cell walls (Fig. 2*e*, *f*). The matrix of the medulla in the wound area was coloured dark blue, perhaps due to a further release of phlorotannins (Fig. 2*f*). In the wound area, the cut cells of the medulla and cortex seemed to bleed out, as the cell walls were colourless (Fig. 2*e*, *f*). After 9 days, the medulla was clearly closed (Figs 2*g*, *h*, 3*a*, *b*). New cell walls indicate cell division activities in the cortex tissue (Fig. 2*h*). The newly formed cells in the medulla and cortex were isodiametric to rectangular, and contained numerous physodes (Figs 2*h*, 3*b–d*), as did the medullary cells behind the wound area (Fig. 3*e*).

Accumulation of phlorotannins

Bright field and fluorescence microscopy of living tissue showed a clear accumulation of phlorotannins in the wound area during the course of the experiment (Fig. 4). In the medulla, a weak phlorotannin fluorescence is already apparent in the 0 d control and is more pronounced after 1 day at the wound surface. Two days after wounding, fluorescence became conspicuous in the medulla adjacent to the wound surface. Over the next few days, especially towards the end of the time series (9 d), fluorescence became even stronger in the cortex tissue at the wound surface and also in the medullary tissue. On day 9, a brown colour in the medulla indicated the presence of phlorotannins in the bright field (Fig. 4). The 9 d control showed no cell divisions and no accumulation of phlorotannins in the wound area but the number of physodes in the cortex appeared to be higher than in the 0 d control.

Influence of radiation conditions

Fluorescence microscopy showed no substantial differences in phlorotannin distribution in the wound area during the 9 days of the experiment between PAR and PAR + UV radiation treatments (data not shown). Also, the structural course of wound healing of *L. hyperborea* was similar and showed the same phases of structural wound healing in PAR conditions and PAR with additional UV radiation.

The number and size of physodes in the epidermis and the outer cortical cells increased significantly during the experiment under PAR + UV radiation as well as under PAR alone, as revealed by staining with toluidine blue O, fast red GG and osmium tetroxide (Fig. 5) (Whitney-Mann U-test, $P = 0.05$). Whereas in the 0 d control physodes were observed mostly in the outermost cortex layer and only occasionally in the adjacent inner cell layer, they regularly occurred in both layers after exposure to PAR as well as after exposure to PAR + UV. In parallel, a higher fluorescence was visible in the epidermis at one side of the blade compared with the epidermis on the other side (Fig. 4).

Discussion

The results of this study show that in the blade of *Laminaria hyperborea* phlorotannins are locally inducible through mechanical wounding. Four different stages of structural wound healing were detected. Additionally, there was an increase in the number of physodes in the outer two layers of the cortex after 9 days exposure to PAR or PAR + UV probably as a protective response to the radiation conditions.

Wound healing and the role of phlorotannins

The wound healing process of *L. hyperborea* can be divided into four stages, similar to the stages described in *E. radiata* (Lüder & Clayton, 2004), and in *Sargassum filipendula* (Fagerberg & Dawes, 1976). The early phase of wound regeneration is characterized by provisional gluing of the wound. The early presence of phlorotannins in *L. hyperborea*, as seen in the fluorescence images (Fig. 4), is evidence of a gluing function at the wound surface to prevent further leaking from the cells. Phlorotannins at the cut surface may initiate a clotting mechanism, involving protein precipitation, which results in the stabilization of proteins and seals the injured region (Sieburth, 1969; Ragan & Glombitza, 1986). Further release of phlorotannins may also prevent bacterial infections (Sieburth, 1969; Ragan & Glombitza, 1986; Iken *et al.*, 2009).

Other ways to prevent further leakage are divisions of the medullary cells, known to occur in *E. radiata* (Lüder & Clayton, 2004), or by closing of the cell connections between wounded and unwounded cells as described in *F. vesiculosus* (Fulcher & McCully, 1969). In *S. filipendula*, the wound closed over to prevent further bleeding; phlorotannins built a plug together with proteins and also functioned as an antifouling and disinfecting complex (Fagerberg & Dawes, 1976).

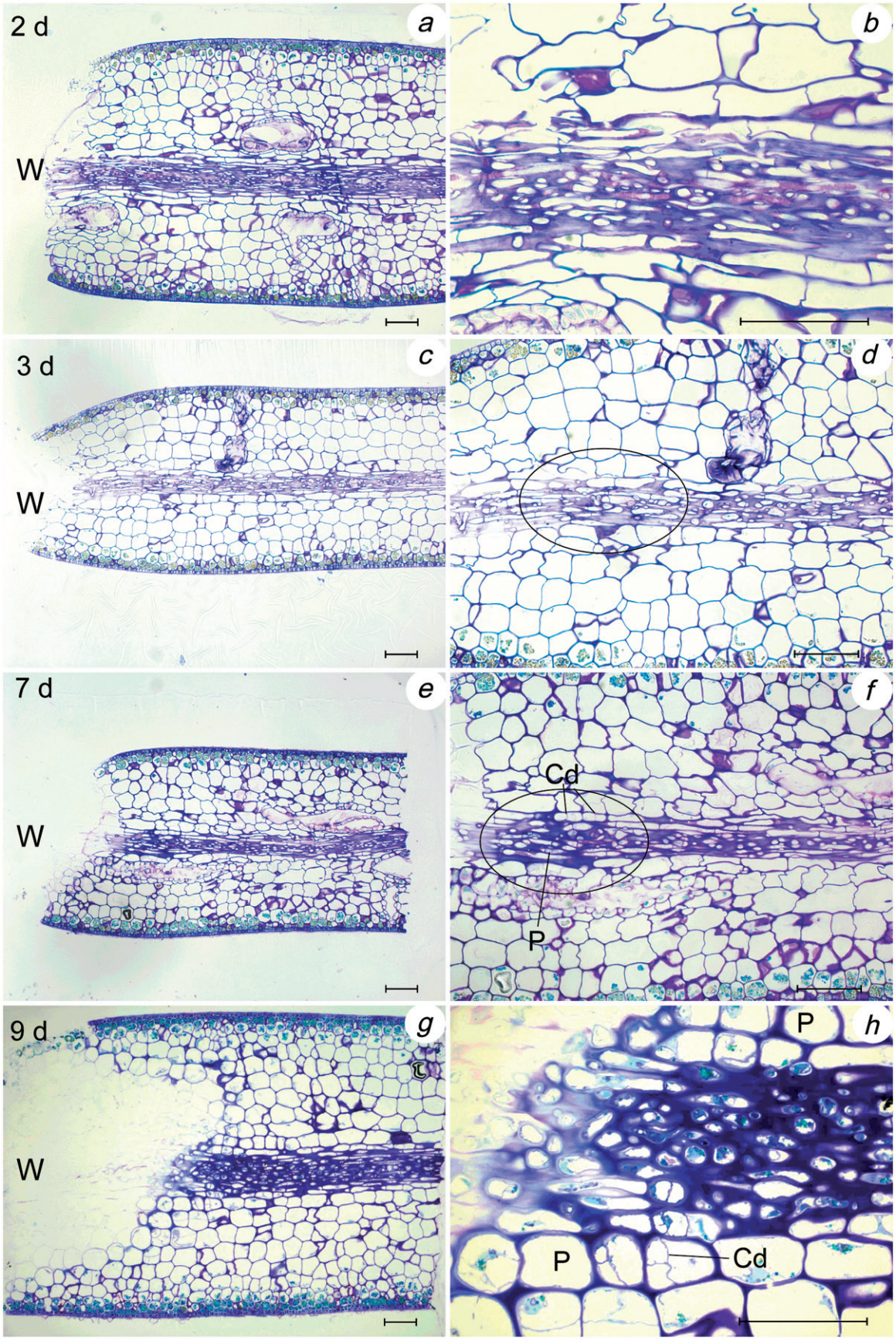

Fig. 2. Structural changes in *Laminaria hyperborea* after wounding; physodes are stained green to blue-green with toluidine blue O; (*a*) overview and (*b*) detailed view of the medulla at the wound surface after 2 days; (*c*) overview and (*d*) detailed view of the medulla at the wound surface with beginning of closure after 3 days; (*e*) overview and (*f*) detailed view of the medulla at the wound surface after 7 days; medulla at the wound surface is a thicker and dark blue, inside the cells are lots of blue-coloured physodes; (*g*) overview and (*h*) detailed view of the closed medulla at the wound surface after 9 days. Abbreviations: Cd: cell division, P: physodes, W: wound site. Scale bars represent 100 µm.

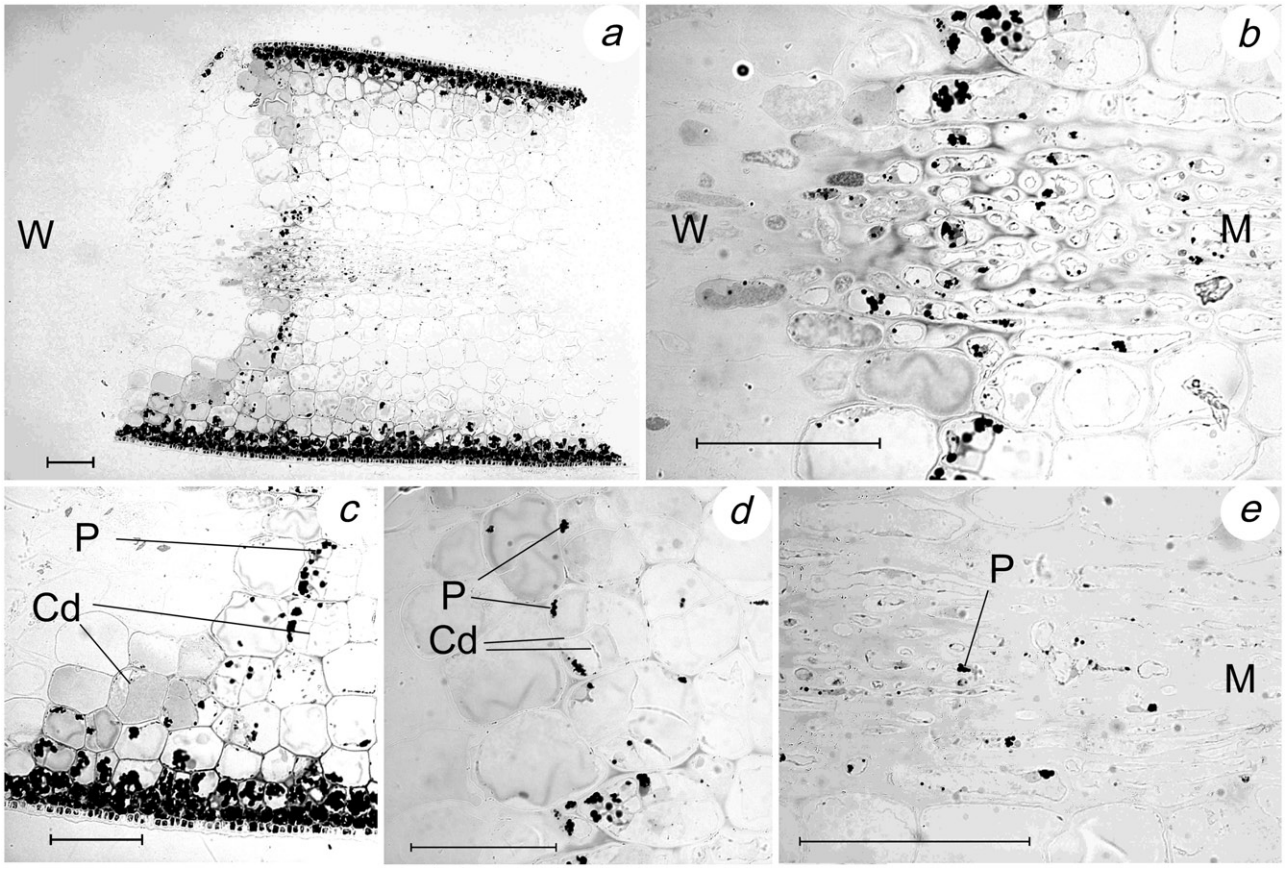

Fig. 3. Structural changes in *L. hyperborea* after wounding. Physodes are stained black with osmium tetroxide; (*a*) overview of the wound healing 9 days after wounding; (*b*) medulla with accumulation of physodes in the wound area; (*c*) cortex showing dividing cells and accumulation of physodes; (*d*) dividing cortical cells containing numerous physodes; (*e*) medulla further away from the wound area. Abbreviations: Cd: cell division, M: medulla, P: physodes, W: wound site. Scale bars represent 100 μm.

The second stage in the regeneration process is characterized by the closing over of the medulla. Transport of assimilates takes place in the medulla, so it is important to stop further loss quickly. The increasing phlorotannin fluorescence at the wound surface from the second day onward and the turquoise colour of the wound area under blue-violet light are interpreted to be signs of phlorotannin leakage, which reacted with other cell constituents or substances in the medium. A similar reaction has been described in *E. radiata* (Lüder & Clayton, 2004).

The third stage is characterized by structural wound healing. The medulla is finally closed by a plug consisting of a denser medullary matrix, characterized by a darker colour in toluidine blue O staining (Fig. 2*d–f*). The cells are filled with phenolic substances and the first cell divisions occur. Likewise, *E. radiata* closes over the medulla with newly formed cells originating from the inner cortex (Lüder & Clayton, 2004). In contrast, in *S. filipendula* a medullary pit is formed by continuous cell division, while medullary cells continuously lose cytoplasm, resulting in shrinkage and collapse of medullary cell walls. Later, a new blade can be formed out of this pit (Fagerberg & Dawes, 1976).

In the cortex of *L. hyperborea* further accumulation of physodes occurred at the wound surface after 5–7 days, and after 9 days in the medulla behind the wound area. In *E. radiata*, polyphenols are first enriched in the medulla, then in the cortex and finally in the medulla behind the wound area (Lüder & Clayton, 2004). In *S. filipendula* physodes are enriched at the wound area, but also accumulate in neighbouring cells after 1 to 2 days (Fagerberg & Dawes, 1976). In *L. hyperborea* it is not clear if the physodes are produced on site or transported. The blue colour of physodes with toluidine blue O staining indicates that the phlorotannins were not freshly produced, which would result in a more greenish colour (Evans & Holligan, 1972), but that they were most probably transported through the medulla (Fig. 2*d*). Such transport has not been described previously. If the physodes are old rather than newly produced, a change in total phlorotannin content may not be measurable. Coupled with the leakage

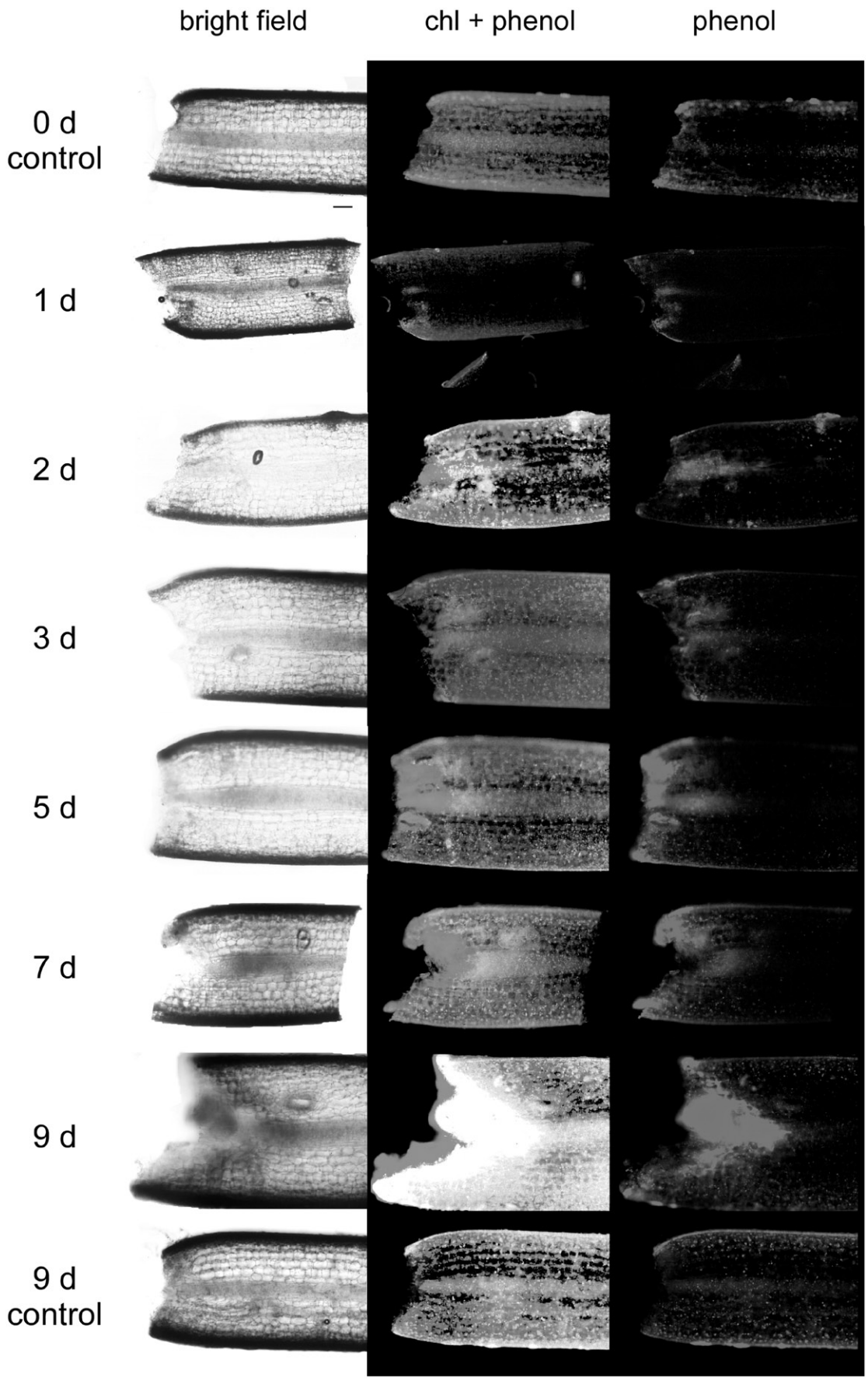

Fig. 4. Time course of wounding and the occurrence of phlorotannin-containing physodes in living tissue of *L. hyperborea*. Phlorotannins are brown to dark under bright field (bright field), fluoresce white and light blue-green under violet-blue light, together with orange fluorescence of chlorophyll (chl + phenol), and green under blue light excitation (phenol). In all images the wound is on the left side. Scale bar represents 100 μm.

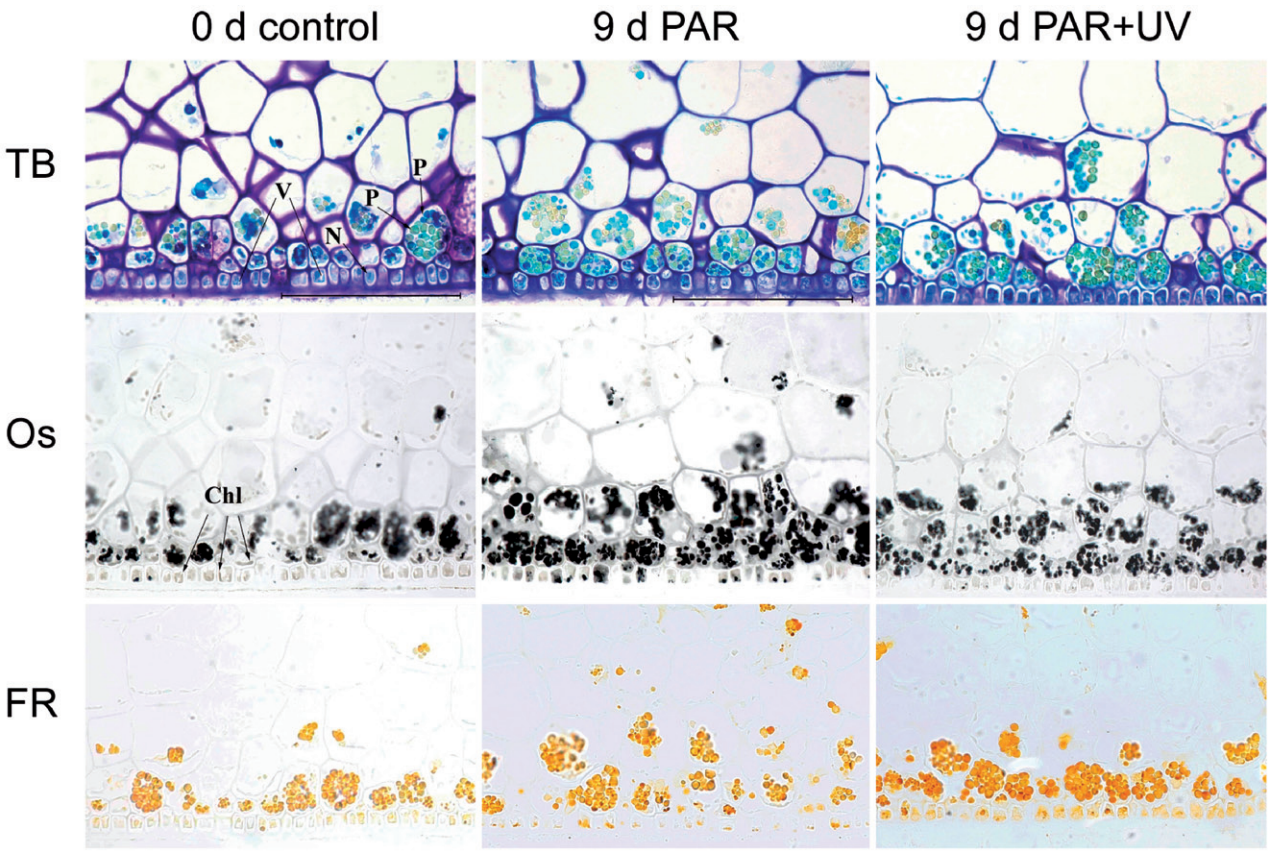

Fig. 5. Comparison of the distribution of physodes in the epidermis and outer cortex of *L. hyperborea* in 0 d control (column 1), after 9 d under PAR (column 2) and after 9 d under UV radiation (column 3) with different stains: toluidine blue O (TB), osmium tetroxide (postfixation, Os) and fast red GG (FR). Abbreviations: Chl: chloroplasts, N: nucleus, P: physodes, V: cell vacuole. Scale bars represent 100 µm.

due to wounding a decrease in phlorotannin concentration would be most likely, as was observed by Toth & Pavia (2002).

Hammerstrom and colleagues (1998) propose that a higher phlorotannin concentration indicates defence against herbivores. Herbivores of *L. hyperborea*, e.g. *Idotea baltica*, *I. emarginata* (Lars Gutow, pers. comm.), are very mobile. The induction of phlorotannins takes several days, and therefore it would not function very effectively against herbivores. The accumulation would only prevent further feeding at the same position at a later point of time. Our results link phlorotannin production mainly to the healing process, suggesting that any anti-herbivore properties may simply be a secondary benefit of this compound.

The final stage of wound regeneration is characterized by cell division and re-differentiation. The new cells are square to rectangular indicating a re-differentiation of the medullary and cortical cells. After 9 days, the wound reaction was not yet complete. We therefore assume that a new epidermis is formed, as shown for *E. radiata* (Lüder & Clayton, 2004).

It can be speculated that cutting the blades into sections may have influenced the resources for formation of phlorotannins. The fluorescence images show that phlorotannins are present from the beginning, and that the wound reaction is spatially restricted to the wound surface and 3–4 cell layers beneath it. An effect of the wounding on the resources for formation of phlorotannins a couple of day earlier cannot be fully excluded but seems unlikely in the eyes of the authors.

Toth & Pavia (2002) were not able to show induction of phlorotannins in *L. hyperborea* by herbivore experiments by measuring the total phlorotannin content, but they found high variation in, or a decrease in phlorotannin. In this study, we were able to show enrichment of phlorotannins in the wound area. A possible explanation for the differences between the studies may lie in the experimental design. Toth & Pavia (2002) showed great variation in total phlorotannin content both between and within individuals and populations. Our overview sections (Fig. 3*a*) showed that most of the physodes are located under the epidermis and not in the area of the wound, so an enrichment

of physodes at the wound is quantitatively not important and not detectable by analytical methods. These differences highlight the importance of microscopic studies.

Protection against excessive UV radiation and PAR through phlorotannins

Ultraviolet radiation has various effects at the organismal and cellular levels. At the organismal level, growth and/or reproduction may be inhibited (Karsten *et al*., 2009). At the cellular level, biological molecules such as DNA may be damaged with deleterious effects (Dring *et al*., 1996; Hanelt *et al*., 1997; Aguilera *et al*., 1999; Roleda *et al*., 2004). Exposure to UV radiation can also inhibit or damage photosynthesis (Dring *et al*., 1996; Hanelt *et al*., 1997; Bischof *et al*., 1998*a*, 1998*b*, 2000) or cause indirect damage through oxidative stress (Aguilera *et al*., 2002). Macroalgae can react to this damage through repair mechanisms (e.g. Poll *et al*., 2003), enzymatic or chemical defence systems (e.g. Aguilera *et al*., 2002), or protection through UV absorbing substances. In brown algae, phlorotannins are proposed to be UV protective compounds due to their absorption maxima in the UV-B (and UV-C) region (Pavia *et al*., 1997; Pavia & Brock 2000; Schoenwaelder, 2002*a*). Protection against excess irradiance was one of the first functions attributed to physodes (Berthold, 1882; McLachlan & Craigie, 1964).

The distribution of physodes after 9 days shows an increase of physodes in the outer cortex as well as in the epidermal cells relative to the 0 d controls. Fluorescence of the epidermis also increased at one side (Fig. 4). Interestingly, this reaction occurs not only under UV conditions, but equally also under exposure to PAR (Fig. 5). It is possible that due to its sublittoral habitat *L. hyperborea* is adapted to lower light conditions than used in the experimental setup. As a consequence, the stronger light conditions in the laboratory (25–30 µmol photons $m^{-2} s^{-1}$) than in the field caused the formation of more physodes in the epidermis and the cortex. Light conditions at the depth and time of the year when samples were collected (10 m, September) approach 0.7–1.4% of surface irradiance, and only 2–5 µmol photons $m^{-2} s^{-1}$ (Lüning & Dring, 1979), which is approximately 10% of the light conditions in the experimental setup. Although phlorotannins mainly absorb in the UV region of the spectrum, some absorption also occurs in the visible part of the spectrum. So we hypothesize that the physodes protect other cell constituents from excessive radiation.

The process of acclimation to new conditions takes about 14 days. We acclimated the algae for 2 days, and the duration of the experiment was 9 days. The algae were therefore acclimating right through the experiment, resulting in an increase of physodes in the outer cortex. The increase in physodes may also be facilitated by an increase in carbon resources due to higher photosynthetic rates.

Observations from preliminary tests showed a high release of phlorotannins into the medium by *L. hyperborea* in contrast to *L. digitata* or *Saccharina latissima* (Halm, unpublished data) under the same conditions. The thin sections of *L. hyperborea* showed broadening of the slime channels which were surrounded by a layer of secretory cells. These cells were characterized by a high physode content, and seemed to release phlorotannins into the slime channels (Fig. 1*a*, *c*). The increased phlorotannin levels are also associated with higher fluorescence in the tissue around the channel (Fig. 4, 3 days). It is known that phlorotannins can be released into the seawater through slime channels to provide UV protection (Fogg & Boalch, 1958; Craigie & McLachlan, 1964; Swanson & Drühl, 2002), but this has not been previously shown at a microscopic level. From this perspective, the release of phlorotannins in our experiments may be a sign of stress under culture conditions. Light-dependent release of phlorotannins has been described in *A. nodosum*, *F. spiralis* and *Pelvetia canaliculata* (Zavodnik & Jensen, 1981). *Macrocystis integrifolia* releases phlorotannins into the seawater, induced by UV-A and UV-B radiation but not by PAR (Swanson & Druehl, 2002).

A similar reaction is seen in spores of *Alaria esculenta* (Wiencke *et al*., 2007), *Laminaria digitata* (Roleda *et al*., 2010) and *Saccorhiza dermatodea* (Roleda *et al*., 2006*a*, 2006*b*). In all cases, exposure to PAR for 8 h leads to enhanced absorption in the UV-B range indicative of phlorotannin release. After additional UV-A and UV-B exposure the absorption in this spectral range decreases, indicating photo-degradation of the phlorotannins. In a parallel study of *Alaria esculenta*, *L. digitata* and *Saccharina latissima*, the absorption by a zoospore suspension was always higher after PAR exposure compared with exposure with additional UV-A and UV-B (Müller *et al*., 2009). This result was, however, not reflected by the actual content of UV-absorbing phlorotannins. However, in their study the spores were exposed only for 8 or 16 h, whereas in the present study the experimental individuals were exposed to radiation for 16 h per day over 9 days. Furthermore, we investigated different

stages in the life history (adult sporophytes rather than spores), which may react differently.

This study further supports the hypothesis that phlorotannins play an important role as a mechanism for repairing damage by grazing and as protective compounds against radiation. Using microscopy techniques, phlorotannins were shown to be inducible by wounding and high PAR and UV radiation. Our results are another example of the multi-functionality of brown algal phlorotannins.

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