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Endogenous and exogenous elicitors of a hypersensitive response in *Gracilaria conferta* (Rhodophyta)

Florian Weinberger[1,2,*] & Michael Friedlander[1]
[1]*Israel Oceanographic & Limnological Research, National Institute of Oceanography, P.O.Box 8030, Haifa 31080, Israel*
[2]*UMR 1931 CNRS, Station Biologique de Roscoff, Place Georges Teissier, 29682 Roscoff, France*

(*Author for correspondence; phone +33-298292361; fax +33-2982923241; e-mail weinberg@sb-roscoff.fr)

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Abstract

Certain forms of oligocellulose and certain bacterially excreted peptides were identified as endogenous and exogenous elicitors, respectively, of a tip bleaching response in *Gracilaria conferta* (Schousboe ex Montagne) J. et G. Feldmann. The half-maximal tip bleaching response was observed when 31.1 μM cellobiose or 11.6 μM cellotetraose were present in the growth medium. In contrast, no response was detected after exposure to glucose, cellotriose, cellopentaose or maltooligosaccharides. The response was thus strongly dependent on the molecular size of the oligocellulose and only saccharides that consisted of an even number of glucose residues were elicitor-active. Three bacterial species that had earlier been identified as potential inducers of the tip bleaching symptom excreted elicitor-active compounds into the growth medium. These compounds were protease-sensitive and thus peptides or proteins. The tip bleaching-inducing compound that was excreted by one *Cytophaga*-like organism was partially purified. It could be extracted from culture supernatants with chloroform and its molecular size was between 700 and 1500 Da, corresponding with a structure of 4–20 amino acids. Various endogenous and exogenous elicitors are thus recognized by *G. conferta* and allow this alga to respond hypersensitively to the maceration of its cell wall skeleton or just to the presence of certain epiphytic organisms.

Introduction

The appearance of thallus depigmentation symptoms in species of the genera *Gracilaria*, *Eucheuma* and *Kappaphycus* after inoculation with certain bacteria has repeatedly been reported (Weinberger et al., 1994; Largo et al., 1995; Jaffray & Coyne, 1996; Weinberger et al., 1997). In agarophytes of the genus *Gracilaria*, agar degrading organisms have been suspected as inducers of thallus whitening, since the injection of such organisms into the thallus of *G. gracilis* resulted in loss of pigmentation (Jaffray & Coyne, 1996). Exposure of *G. conferta* to oligoagars resulted in an oxygen activation and subsequently in thallus tip bleaching and elimination of associated microorganisms (Weinberger et al., 1999). These findings strongly indicated that the oligosaccharide products of bacterial enzymatic attacks on the agar cell wall matrix resulted in a defense response by *G. conferta*.

It is well known that spermatophytes respond to pathogen attacks with a 'hypersensitive' response, culminating eventually in the induction of defense related enzymes or synthesis of additional cell wall materials. Hypersensitive responses are triggered by elicitors, chemical signals that are recognized by the plant cell and indicate the presence of pathogens to it (Dixon & Lamb, 1990; Kombrink & Somssich, 1995; Hahn, 1996). Endogenous elicitors are anabolic products of the host. An example are the oligopectins, which are released from host cell walls due to the hydrolysing action of pathogen pectinase (Nothnagel et al., 1983). Exogenous elicitors, in contrast, are anabolic products

of the pathogen. For example, they may be constituents of its outer membrane (Graham et al., 1977) or cell wall (Sharp et al., 1984; Baureithel et al., 1994) or they may be excretions (e.g. Parker et al., 1991; Wei et al., 1992; Nürnberger et al., 1994). A main characteristic feature of hypersensitive responses is the fast formation of defense necroses: host cells that are close to an intruding pathogen die and their decompartimentation is typically followed by accumulation of toxic defense agents that contribute to the death of the pathogen (Dangl et al., 1996; Gilchrist, 1998). The observations that have been made so far strongly suggest thallus tip bleaching of *G. conferta* to be a manifestation of the algal hypersensitive response and thus an indication of elicitation, rather than of disease (Weinberger et al., 1999).

Several strains of thallus tip bleaching inducing bacteria have been isolated from *G. conferta* in the past, which could be assigned to three different species. Of these, two species were related to the *Flavobacterium-Cytophaga* group and one to the *Corynebacterium-Arthrobacter* complex (Weinberger et al., 1997). Only one of these species degraded agar (Weinberger et al., 1997). Thus it appears that oligoagars can not be the only signal for the hypersensitive response. The purpose of this study was to identify further elicitors of thallus tip bleaching in *G. conferta*, based on the two expectations that elicitor-active bacteria might produce exogenous elicitors (Weinberger et al., 1997) and that the algal cell wall skeleton might be a source of endogenous elicitors.

Material and methods

Preparation of bacteria and purification of bacterially excreted elicitor-active compounds

Corynebacterium-Arthrobacter 1, that had been isolated from *G. conferta* and characterized earlier (Weinberger et al., 1997), were cultivated in liquid medium or on nutrient agar (Weinberger et al., 1997) at 25 °C in darkness. Cells and medium of liquid cultures were separated by centrifugation at 12,000 × g for 1 h. Sterility of the resulting supernatants was verified by incubation of 100 μL on nutrient agar for at least three days. The selected strains developed macroscopically visible colonies in this period of time, when they were present in the supernatant. In order to destroy proteins in the supernatants, they were treated with 100 U mL^{-1} pronase (E.C. 3.4.24.31) and the mixture was incubated at 25 °C for 3 h. The active compounds were separated from cell free supernatants by extraction for 30 min with twice as much volume of solvent in a separation funnel. The aquatic phase was frozen and lyophilized; the non-polar phase was dried in an evaporator at room temperature. After lyophilization and dilution in 1 mL distilled water, elicitor active residues were further purified by size exclusion on Sephadex G-15 and Sephadex G-10 columns, using NaCl (1M) and distilled water (as solvents) for elution, respectively. The eluates were monitored with a flow-through photometer at 280 nm and eluate fractions were taken as soon as increasing optical density indicated the beginning elution of proteins. Aliquots of all purification steps were diluted in distilled water and stored in a freezer (–80 °C).

Preparation of saccharides

For partial acid hydrolysis, 0.5 g cellulose powder was dissolved in 8 mL trifluoroacetic acid and incubated for 15 or 30 min at 80 °C in closed screw cap tubes. The hydrolysate was lyophilized and remaining trifluoroacetic acid was removed by three consecutive cycles of dilution in methanol and vacuum evaporation at 40 °C. A control preparation was made in which trifluoroacetic acid was incubated without addition of cellulose. The certified purities of commercial oligosaccharides were $>$ 98%. Stock solutions were made up from all saccharides, adjusted to pH 8.0 and a salinity of 3.5%, filter sterilized (0.2 μm mesh width) and kept in a freezer (–80 °C). The content of reducing sugars in the stock solutions was determined photometrically with the neocuproine assay (Dygert et al., 1965) and regarded as an indication of their molarity.

Preparation of algae and tip bleaching assay

G. conferta was incubated in test tubes at 25 °C on a shaker (100 strokes min^{-1}) and exposed to 12.5 h of artificial light (cool white, 40 μmol photon m^{-2} s^{-1}) per day. All experiments were performed with apparently healthy *G. conferta* plants that originated from outdoor tanks. The algae underwent a pretreatment incubation of three days with the antibiotics Vancomycin and Cefotaxim at 100 mg mL^{-1} each. This treatment eliminates epiphytic bacteria, represses thallus tip bleaching and does not affect *G. conferta* negatively (Weinberger et al., 1997). After the pretreatment, *G. conferta* was exposed for 16 h to test conditions by discarding the old medium and replacing with fresh sterile medium. Bacterial supernatants,

Table 1. Impact of cell-free culture fluid of 13 bacterial isolates on the approximate relative risk of tip bleaching in *G. conferta*. Control groups had been exposed to sterile liquid medium when the effect of untreated culture supernatants was investigated. They had been exposed to untreated supernatants when the effect of protease-treated supernatants was examined. * = significant difference from control ($p \leq 0.05$). ** = significant difference from control ($p \leq 0.01$)

Strain		Untreated culture supernatants			Protease treated culture supernatants		
		Approximate relative risk of tip bleaching	95% confidence interval	(n controls/ n treatments)	Approximate relative risk of tip bleaching	95% confidence interval	(n controls/ n treatments)
Flavobacterium-Cytophaga 1	A	3.6**	2.3–5.6	(282/205)	0.06**	0.01–0.4	(38/42)
	B	2.9**	1.5–5.5	(219/134)	0.09**	0.02–0.5	(74/59)
	C	2.2**	1.5–3.3	(504/327)	0.39**	0.2–0.7	(205/160)
	D	1.9*	1.1–3.5	(170/200)	0.53	0.08–3.5	(105/63)
	E	4.3**	2.3–7.9	(360/187)	0.17**	0.06–0.5	(48/43)
Flavobacterium-Cytophaga 3	A	2.5**	1.3–5.1	(94/93)	0.37**	0.2–0.8	(65/93)
Corynebacterium-Arthrobacter 1	A	2.0*	1.1–3.7	(117/128)	0.47	0.2–1.1	(62/54)
	B	3.0**	1.8–4.9	(154/181)	0.33**	0.2–0.6	(82/91)
	C	2.7**	1.5–4.7	(74/280)	0.39**	0.2–0.6	(280/102)
	D	2.8**	1.6–5.1	(74/188)	0.21**	0.09–0.5	(188/94)
	E	2.6**	1.4–4.8	(74/164)	0.43**	0.2–0.7	(164/99)
	F	3.7**	2.1–6.5	(74/214)	0.13**	0.07–0.2	(214/116)
	G	3.5**	2.2–5.8	(154/241)	0.37**	0.2–0.6	(241/172)

their purification products or saccharides were added from stock solutions. Bleached and non-bleached tips were counted before and after incubation under test conditions as described earlier (Weinberger et al., 1997). The ratios of bleached and non-bleached tips were determined for treatments and controls. Approximate relative risks of bleaching were then calculated from the odd ratios of treatment and control groups and tested for significance with the χ^2-test according to Fisher and Van Belle (1993). The approximate relative risk in control groups is defined as 1. Approximate relative risks > 1 and < 1 thus indicate that treatments resulted in more and less bleaching than control treatments, respectively. For the iterative computation of dose-response curves, tip bleaching was calculated for each replicate by subtraction of the relative amounts of bleached tips after and before exposure to test conditions. Finally, tip bleaching in control treatments was subtracted from tip beaching in test conditions. This calculation resulted in the 'bleaching-impact' (BI), which indicated the tendency of tips to undergo bleaching, as compared to control treatments. A negative BI indicates that more bleaching was detected in the control samples than in treated samples. The logistic formula used to describe dose-response kinetics was

$$BI = MAX^*(1 + 10^{-SLOPE^*(log(X) - log(EC50))})^{-1}$$

(Hahn et al., 1996).

In this equation, MAX represents the maximum response and EC_{50} is the dose necessary to obtain 50% of MAX. The parameter SLOPE describes the average dose-dependent increase in the algal response.

Results

Cell-free supernatants from liquid cultures of 13 bacterial strains caused significant loss of pigments in *G. conferta* tip cells (Table 1). The approximate relative risk of tip bleaching after addition of these supernatants to cultures of *G. conferta* could be reduced in most cases by a treatment with protease (Table 1). The reduction was not significant for two strains, *Flavobacterium-Cytophaga* 1D and *Corynebacterium-Arthrobacter* 1A. The approximate relative risk after addition of untreated culture supernatants of these two isolates was already relatively low, and it was subsequently difficult to detect the further reduction by protease treatment with $p \leq 0.05$.

Table 2. Impact of different concentrations of cellulose microcrystals unhydrolysed and hydrolysed with trifluoro-acetic acid (TFA) on the approximate relative risk of tip bleaching in *G. conferta*. TFA residue without cellulose was tested at the same concentrations that were present in the cellulose hydrolysate. The sample size of the control group (addition of water only) was n = 47. * = significant difference from control ($p \leq 0.01$)

	Cellulose concentration [mg L^{-1}]	Residue dilution [L^{-1}]	Approximate relative risk of tip bleaching	95%-confidence interval	n
Cellulose	500	–	1.1	0.1–10.7	44
	100	–	1.8	0.2–14.4	44
	50	–	1.6	0.2–12.9	49
	10	–	1.1	0.1–11.2	42
Residue of cellulose	500	1	49.0*	9.0–267.0	70
after treatment with	100	0.2	20.4*	3.5–119.3	33
TFA	50	0.1	17.6*	3.1–101.3	39
	10	0.02	8.0*	1.4–46.1	55
Residue of TFA	0	1	1.2	0.1–11.8	40
	0	0.2	0.4	0.0–11.0	35
	0	0.1	0.6	0.0–14.9	26
	0	0.02	3.3	0.5–23.8	35

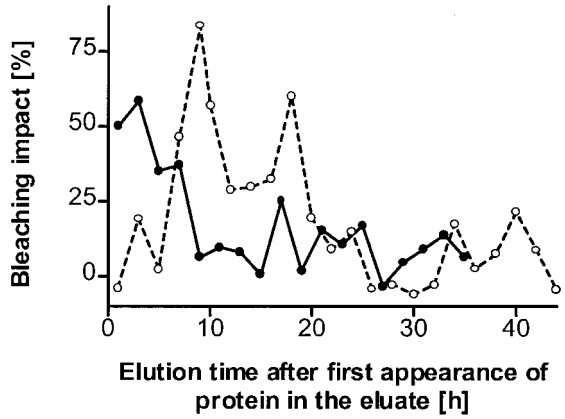

Figure 1. Tip bleaching in *G. conferta*. The chloroform extraction residue of *Flavobacterium-Cytophaga* 1A culture supernatant was separated by size exclusion chromatography elution time and aliquots of the eluates were added to the algal medium. The residue was first separated on Sephadex G-15 (○). The resulting bleaching-inducing fractions were then separated on Sephadex G-10 (●). Each point represents the bleaching impact that was detected in five replicates.

Chloroform proved to be useful for the extraction of the *Flavobacterium-Cytophaga* 1-inducing agent from culture supernatants. For example, the cell free supernatant of isolate *Flavobacterium-Cytophaga* 1A induced 41.9% of bleached tips. After an extraction of 100 mL of the supernatant with chloroform, the water- and organic solvent-fractions induced 7.1% and 34.0% of bleached tips, respectively, when they had been lyophilized and resuspended in 100 mL distilled water. After extraction with chloroform, the *Flavobacterium-Cytophaga* 1-inducing agent was further purified by size exclusion chromatography. Fractions that were collected 8 to 18 h after the first elution of proteins from Sephadex G-15 had an increasing impact on tip bleaching (Figure 1) and thus contained the agent. Further purification of these fractions on Sephadex G-10 resulted in immediate elution of the tip bleaching inducing agent (Figure 1). The signal was thus excluded by Sephadex G-10 (fractionation range < 700 Da), but not by Sephadex G-15 (fractionation range < 1500 Da), which indicated a molecular weight between 700 and 1500 Da.

Not only bacterial isolates and their excretions, but also potential products of the hydrolysis of the algal cell wall skeleton induced thallus whitening in *G. conferta*. Cellulose that had been partially hydrolysed with trifluoroacetic acid (TFA) increased the approximate relative risk of tip bleaching significantly at 0.01 g L^{-1} (Table 2). The effect could neither be induced with unhydrolysed cellulose, nor with control preparations that had been made with TFA only, and was thus due to the cellulose hydrolysate. A significant bleaching inducing effect was also found with commercial cellulose degradation products, but only

Table 3. Impact of mono- and oligosaccharides on the approximate relative risk of tip bleaching of *G. conferta*. Controls were treated with sterile water. * = significant difference from control ($p \leq 0.01$)

Saccharide	Approximate relative risk of tip bleaching	95%-confidence interval	(n controls / N treatments)
D-Glucose (500 μM)	1.0	0.1–7.0	(47/110)
Cellobiose (100 μM)	6.2*	2.7–14.3	(125/62)
Cellotriose (100 μM)	1.6	0.9–3.0	(138/118)
Cellotetraose (100 μM)	6.6*	3.8–11.6	(239/81)
Cellopentaose (100 μM)	0.7	0.3–1.6	(138/77)
Maltose (100 μM)	1.0	0.4–2.7	(138/38)
Maltotetraose (100 μM)	1.2	0.6–2.4	(101/59)
Lactose (500 μM)	1.3	0.5–3.3	(107/77)
Sucrose (500 μM)	0.8	0.3–2.7	(78/39)
Stachyose (500 μM)	0.4	0.1–1.4	(107/65)

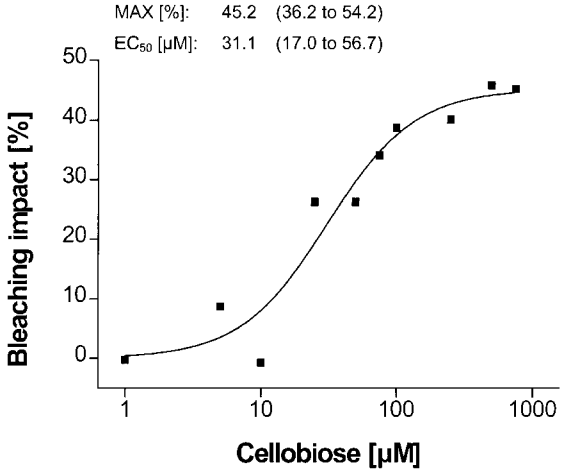

Figure 2. Bleaching impact of cellobiose. Each symbol represents between 28 and 71 tips. The constants computed for the best-fitting logistic function (r^2 = 0.939) are given with the 95% confidence interval in the figure.

with oligosaccharides that consisted of an even number of glucose-residues – cellobiose and cellotetraose (Table 3). No effect could be detected with products of cellulose degradation that consisted of an odd number of residues (cellopentaose, cellotriose and D-glucose). Maltose or maltotetraose, saccharides that are different from cellobiose and cellotetraose because they are linked $\alpha(1\rightarrow4)$ and not $\beta(1\rightarrow4)$, had also no impact on bleaching, and the same was found for several other mono- and oligosaccharides that were tested (Table 3). The bleaching impact of cellobiose (Figure 2) and cellotetraose (Figure 3) was clearly dose-dependent.

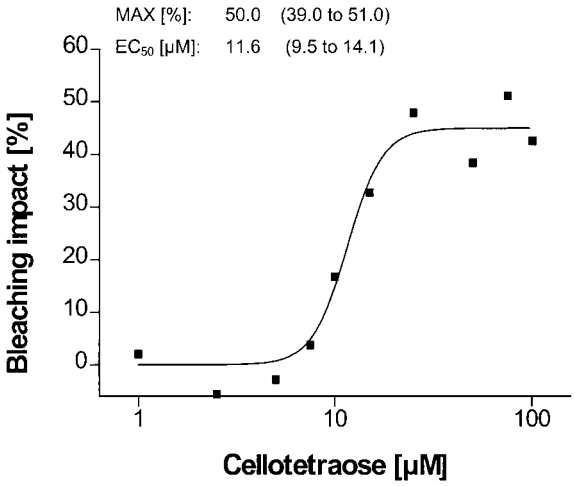

Figure 3. Bleaching impact of cellotetraose. Each symbol represents between 27 and 108 tips. The constants computed for the best-fitting logistic function (r^2 = 0.963) are given with the 95% confidence interval in the figure.

Similar maximal bleaching impacts were computed for both saccharides, but the algal sensitivity to cellotetraose was 3 times higher, as can be seen from the lower dose required to induce 50% of the maximal response.

Discussion

Cellulose hydrolysis products and certain bacterially excreted peptides are in this study shown to induce the thallus tip bleaching symptom in *G. conferta* that can also be elicited with oligoagars (Weinberger et al., 1999). The identical algal response after exposure to these agents indicates that they also induce an autodestructive oxygen activation.

The cell wall skeleton of *Gracilaria* consists mainly of a cellulose-type polymer with xylose and mannose side chains (Bellanger et al., 1990). It is sensitive to cellulase (Bellanger et al., 1990; Björk et al., 1990) and cellulose degradation products are therefore potential endogenous elicitors in the same sense as agar degradation products. Eventually, enzymatic cellulose degradation takes places in the cellwall of living *Gracilaria* after a successful invasion of certain fungi or bacteria. Such organisms were occasionally reported from seaweeds (Kong & Chan, 1979; Ramaiah & Chandramohan, 1992). Cellulose degrading fungi often release cellulase into their medium and products of these enzymes may then accumulate (Coughlan, 1985). This has also been observed with

plant pathogens such as *Rhizoctonia solani* or *Botrytis cinerea* and with several marine fungi (Kohlmeyer & Kohlmeyer, 1979).

Cellulose microcrystals had no eliciting effect on *G. conferta*, probably due to the fact that they are not water soluble and too large to diffuse into the cell wall free space and interact with the cell. Elicitor activity was detected only with cellulose hydrolysate (Table 2) and the algal response to cellulose degradation products was highly specific. Cellobiose and cellotetraose were active, but not maltose and maltotetraose (Table 3), although all four saccharides consist of D-glucose residues. The residues in malto-oligosaccharides are $\alpha(1\rightarrow4)$-linked, while cello-oligosaccharides are characterized by $\beta(1\rightarrow4)$-linkages. This difference results in distinct steric structures of malto- and cello-oligosaccharides, which apparently affect the algal ability to react.

Another factor was the size of cello-oligosaccharides. Three times more cellobiose (31 μM, Figure 2) than cellotetraose (11 μM, Figure 3) was necessary to induce 50% of the maximal tip bleaching reaction in *G. conferta*. Only these oligocelluloses, both consisting of an even number of saccharide residues, induced bleaching. Cellotriose and cellopentaose, consisting of an odd number of saccharide residues, had no effect (Table 3). A high specificity that varies with varying molecule sizes is typical for oligosaccharide receptor systems in plants. For example, the optimum-sized β-glucan, oligochitin and oligogalacturonide elicitors of defense-related responses in spermatophyte cells were a heptasaccharide (Sharp et al., 1984), a pentasaccharide (Baureithel et al., 1994) and saccharides consisting of at least 9 monosaccharide residues, respectively (Nothnagel et al., 1983, Jin & West, 1984, Bishop et al., 1984). Oligoagars with more than 8 residues were optimum-sized elicitors of tip bleaching in *G. conferta* (Weinberger, unpublished). The size-specificity of the algal response after exposure to oligocelluloses suggests that a receptor system for these saccharides exists.

Not only endogenous, but also exogenous elicitors induced the tip bleaching responses in *G. conferta*. Thirteen bacterial isolates of at least three different species that were tested produced agents that caused thallus tip bleaching in *G. conferta*, confirming the result of an earlier study (Weinberger et al., 1997). In eleven cases these agents could be destroyed with protease (Table 1) and were therefore peptides or proteins. However, they were no host cell wall degrading enzymes. Cellulase played no role because the inducing bacterial strains were unable to hydrolyse cellulose and produced no cellulase (Weinberger et al., 1997). Agarase could be excluded for similar reasons, because only *Flavobacterium-Cytophaga* 1 was able to hydrolyse agar (Weinberger et al.,1997). The tip bleaching inducing agent excreted by this species had a molecular weight of only 700–1500 Da (Figure 1), probably a peptide of only 4 to 20 amino acids, and thus too small for an agarase. Several exogenous elicitors of spermatophyte defense responses have been identified as peptides (Scholtens-Toma & De Wit, 1988, Nürnberger et al., 1994), proteins (Wei et al., 1992) or glycoproteins (Stekoll & West, 1978, De Wit & Roseboom, 1980, Parker et al., 1991).

In conclusion, not only oligoagars, but also oligocelluloses cause a tip-bleaching response by *G. conferta*, along with peptide- or protein-excretions of at least three different bacterial species. Various molecular signals of endogenous and exogenous origin are thus recognized by *G. conferta* and allow this alga to respond hypersensitively to the maceration of its cell wall or just to the presence of certain epiphytic organisms.

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