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RESEARCH PAPER

Apoplastic oxidation of L-asparagine is involved in the control of the green algal endophyte *Acrochaete operculata* Correa & Nielsen by the red seaweed *Chondrus crispus* Stackhouse

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Abstract

Gametophytes of the marine alga *Chondrus crispus* are more resistant than tetrasporophytes to infection by the filamentous endophytic alga *Acrochaete operculata*. It has been shown recently that carrageenan oligosaccharides from the resistant gametophytic generation of *C. crispus* stimulate the secretion of L-asparagine (L-Asn) by the endophyte and that the host generates hydrogen peroxide and 2-oxo-succinamic acid after contact with this amino acid. Here the response of *C. crispus* to L-Asn and its effect on the pathogen is investigated. *Chondrus crispus* released hydrogen peroxide, ammonium ions, and a carbonyl compound into the medium when exposed to L-Asn. This response was correlated with an increase in oxygen consumption. Inhibitor studies indicated the involvement of a flavoenzyme in the reaction, which was sensitive to high concentrations of the reaction product, ammonium, and to chlorpromazine, quinacrine, and cyanide, inhibitors of L-amino acid oxidase. Cell wall macerate of *C. crispus* also responded to L-Asn, while protoplasts were inactive. Uptake of L-Asn into the cell was not necessary for the response, suggesting that the involved L-amino acid oxidase is apoplastic. *Acrochaete operculata* was more sensitive to hydrogen peroxide than *C. crispus* and settlement of *A. operculata* zoospores on *C. crispus* was reduced by 86% in the presence of L-Asn. This reduced settlement could be prevented with catalase. *Chondrus crispus* thus features an apoplastic amino acid oxidase, which is involved in the control of its endophytic pathogen. The modulation of the amino acid secretion in *A. operculata* by carrageenan oligosaccharides is therefore a key issue in the etiology of the association.

Key words: *Acrochaete*, asparagine, *Chondrus*, endophytism, host–pathogen interactions, L-amino acid oxidase.

Introduction

Release of activated oxygen species such as hydrogen peroxide (H_2O_2) is a ubiquitous characteristic of plant defence reactions. Active oxygen species may have direct cytotoxic effects on plant pathogens (Mellersh *et al.*, 2002). They also orchestrate a variety of secondary responses, including cell wall cross-linking and synthesis of pathogenesis-related proteins and phytoalexins (Lamb and Dixon, 1997; Wojtaszek, 1997; Bolwell, 1999; Mellersh *et al.*, 2002). In most plant–pathogen interactions, a diphenylene-iodonium (DPI)-sensitive (O'Donnell *et al.*, 1993), membrane-located, and receptor-activated NADPH-oxidase generates superoxide radicals (Levine *et al.*, 1994; Doke and Miura, 1995; Lamb and Dixon, 1997; Bolwell *et al.*, 1998), which eventually dismutate into H_2O_2 and O_2 (Sutherland, 1991). Apoplastic peroxidases (Bolwell *et al.*, 1998; Martinez *et al.*, 1998), as well as various oxidases such

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as oxalate oxidase (Zhang *et al.*, 1995; Thordal-Christensen *et al.*, 1997) or amine oxidase (Laurenzi *et al.*, 2001; Rea *et al.*, 2002), have also been identified as sources of activated oxygen species in higher plants.

Elicitor- or stress-activated, DPI-sensitive oxidases, presumably membrane-bound NADPH oxidases, have been reported in *Chondrus crispus* (Bouarab *et al.*, 1999) and other red algae (Weinberger *et al.*, 1999), as well as in brown algae (Küpper *et al.*, 2001; Coelho *et al.*, 2002). The reactions catalysed by these enzymes were shown to control infections by pathogens, including bacteria (Weinberger and Friedlander, 2000; Küpper *et al.*, 2001) and endophytic filamentous algae (Bouarab *et al.*, 1999; Küpper *et al.*, 2002). However, and even though more evidence for induced defence reactions of algae upon pathogen recognition is emerging (for reviews, see Potin *et al.*, 2002; Steinberg and de Nys, 2002), the host–microbe interactions of most marine algae remain poorly understood.

The interaction between *C. crispus* and its endophytic green algal pathogen *Acrochaete operculata* provides an interesting system to decipher the biochemical bases of host immunity and pathogen virulence in marine algae. The tetrasporophytic and gametophytic life stages of *C. crispus* live in mixed populations in the same environment and are isomorphic, but differ in their susceptibility to *A. operculata*. In infection trials, tetrasporophytic thalli were successfully infected by approximately 10 times more pathogenic zoospores than gametophytes and the spore settlement was patchier on gametophytes than on tetrasporophytes (Correa and McLachlan, 1991; Bouarab *et al.*, 1999). After successful penetration of the outer cell wall, filaments of *A. operculata* developed faster in tetrasporophytic hosts and the penetration of the medulla was a rare exception in gametophytes, but was the rule in tetrasporophytes when host and pathogen were cultivated together for a period of 2 months (Correa *et al.*, 1988; Correa and McLachlan, 1991, 1992, 1994). Gametophytes of *C. crispus* are thus more resistant to *A. operculata* than tetrasporophytes, although no difference was detected in the number of individuals of both life stages of *C. crispus* that were associated with *A. operculata* in natural populations from the British Isles (Plumb, 1999).

The host cell wall matrix polysaccharides of *C. crispus*—κ- and λ-type carrageenans, respectively, in gametophytes and tetrasporophytes—were shown to control endophyte penetration, with λ-carrageenans increasing and κ-carrageenans reducing the endophyte virulence (Bouarab *et al.*, 1999). Contact of *A. operculata* with κ-carrageenans induces enhanced secretion of the nitrogen storage compound L-asparagine (L-Asn), which in turn induces a release of H_2O_2 by *C. crispus* (Weinberger *et al.*, 2002). Here a detailed characterization of the processes, which occur after contact of the host with L-Asn secreted by the pathogen, is provided with focus on the enzymatic H_2O_2 production and its role in the chemical defence of *C. crispus*. It is shown that L-Asn is the substrate of an apoplastic L-amino acid oxidase in the host that generates H_2O_2. The H_2O_2 concentrations generated by *C. crispus* in the presence of physiologically relevant amounts of L-Asn were sufficient to prevent settlement of *A. operculata* zoospores significantly, and it is argued that the host L-amino acid oxidase is involved in the control of the early stages of the infection by *A. operculata.*

Materials and methods

Plant material and cultivation methods

The material of *Chondrus crispus* Stackhouse used was either unialgal and clonal or non-unialgal and non-clonal (='wild type'). Reproductive wild-type material was collected at Pointe St-Barbe, Roscoff, France, separated into gametophytes and tetrasporophytes, and maintained in tanks of running sea water. Wild-type material from Spillanes Cove, New Brunswick (Canada) provided by T Chopin was also used and treated in the same way. Unialgal cultures of *Acrochaete operculata* Correa and Nielsen (isolate KH 04068-1-1 from Ketch Harbour, Nova Scotia, Canada) and of tetrasporophytic *C. crispus* (isolate JC 0001 PC-S from Peggy's Cove, Nova Scotia, Canada) were provided by J Correa and grown in SFC medium prepared from sterile sea water as described previously (Bouarab *et al.*, 1999).

Bioassays

During all experiments, *C. crispus* was incubated at a density of 50 g l^{-1}. The cell wall of *C. crispus* was macerated and protoplasts were prepared according to Le Gall *et al.* (1990). The protoplasts were used at a density of 4.24×10^8 ml^{-1}. For infection trials, three freshly cut fragments of unialgal *C. crispus* were cultivated in 10 ml of SFC medium (Correa and McLachlan, 1991) and inoculated with 4×10^4 ml^{-1} zoospores from *A. operculata.* They were maintained in SFC medium with or without L-Asn (165 μM) and with or without catalase (1100 U ml^{-1}). The culture medium was changed after 24 h and from then on three times a day. After 5 d all spores that had settled on *C. crispus* were counted with a light microscope. The size of *C. crispus* surfaces was determined by image analysis of projections generated with a digital scanner, using the free UTHSC-SA ImageTool software (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the internet at ftp://maxrad6.uthscsa.edu).

For determination of their sensitivity to H_2O_2, various concentrations of H_2O_2 were added to the medium of *C. crispus* and *A. operculata*. The medium was discarded after 60 min and replaced with fresh medium containing 0.05% of the membrane-impermeable stain Evans blue. The staining solution was removed after 30 min and the algae were washed three times with fresh medium. Stained epidermal cells of *C. crispus* and stained cells of *A. operculata* were counted using a light microscope. In addition, stained biomass of *A. operculata* was suspended in a defined volume of sea water and the optical density at 680 nm was measured with a spectrophotometer.

For quantification of the bacterial flora associated with *C. crispus*, the algae were homogenized with an Ultra-Turrax in a defined volume of sterile sea water. The homogenate was then plated out on nutrient agar 2216 (Oppenheimer and ZoBell, 1952) and incubated for 2 weeks at 15 °C in darkness. The numbers of bacterial units that had formed colonies were then counted.

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Monitoring metabolization and uptake of amino acids

In these experiments, 250 mg FW of *C. crispus* were incubated on a shaker in Petri dishes containing 5 ml of autoclaved sea water. Amino acids were added from stock solutions in water. Unless otherwise indicated, all amino acids and other reagents used were from Sigma-Aldrich-Fluka (St Louis, MO, USA). Subsequent H_2O_2 release was quantified using the luminol-assay as described in Bouarab *et al.* (1999). In addition to the treatments of *C. crispus* in medium (a), three different controls were examined in all experiments in order to allow for compensation of H_2O_2 scavenging and H_2O_2 release due to uncontrolled effects. These were (b) medium+ treatment, (c) medium+*C. crispus*, and (d) medium only. The netto-release of H_2O_2 was then calculated according to the formula

$$[(a-b)-(c-d)]\times(\text{algal density})^{-1}=\text{release}$$

Carbonyl compounds (Soda, 1967) and ammonium (Koroleff, 1983) were analysed spectrophotometrically, using oxalacetic acid and NH_4Cl as external standards, respectively; these experiments were controlled in the same way as described for H_2O_2 measurements. In dose–response experiments, *C. crispus* was incubated for 45 min in the presence of L-Asn before H_2O_2, ammonium (NH_4^+) or carbonyl compounds were quantified.

The ability of *C. crispus* to sequester H_2O_2 was monitored by the addition of 20 μM H_2O_2 to the medium and quantification of H_2O_2 remaining after an incubation period of 15 min. Algal O_2 consumption was determined using a stirred temperature-controlled chamber with an integrated oxygen electrode (15 °C; Hansatech Instruments Ltd, King's Lynn, UK), as described earlier (Weinberger *et al.*, 1999).

For investigation of the uptake of L-Asn from sea water by *C. crispus*, L-Asn containing 0.0425% [^{14}C]L-Asn was added to the water. The initial specific activity of the batch was 0.0935 mCi mmol^{-1}. After 1 h of incubation *C. crispus* was rinsed five times for 5 s in 5 ml of fresh sterile sea water in order to remove attached L-Asn from the algal surface. The biomass was then solubilized with perchloric acid–H_2O_2, following Lobban (1974). Radioactivity in the solubilized biomass, as well as in sea water samples taken during incubation, was measured with a Tri-Carb 1500 counter (Packard, Rungis, France).

Pharmacological analysis

Several compounds were screened for their potential activating or inhibiting activity on H_2O_2 generation after exposure of *C. crispus* to 165 μM L-Asn. Ammonium chloride, potassium chloride (final concentration in the medium 100 mM), calcium chloride (100 mM), potassium cyanide, diethylpyrocarbonate (100 μM), and imidazole (20 mM) were added from stock solutions dissolved in H_2O. Quinacrine, chlorpromazine, diphenylene-iodonium (100 μM), salicylhydroxamic acid (10 μM), anthracene-9-carboxylic acid (100 μM), 4-aminopyridine (1 mM), staurosporine (20 μM), 2,4-dinitrophenol (100 μM), and A23187 (50 μM) were added from stock solutions dissolved in dimethyl sulphoxide (DMSO). Kryptofix 222 was purchased from Merck (Darmstadt, Germany) and added from a stock solution in water. Valinomycin (5 μM) and verapamil (100 μM) from Calbiochem (France Biochem, Meudon, France) were added from stock solutions in water and DMSO, respectively.

Results

Metabolization of L-Asn by C. crispus

Not only H_2O_2, but also carbonyl compounds and NH_4^+ accumulated in the medium after exposure of *C. crispus* gametophytes to L-Asn (Fig. 1). H_2O_2 concentrations exhibited a transient maximum after 45 min (Fig. 1A), while maximal carbonyl concentrations were reached after 2 h (Fig. 1B) and remained stable during the next 12 h. NH_4^+ concentrations (Fig. 1C) were generally lower than the carbonyl concentrations and, at 2 h after the addition of L-Asn, they started to decrease, down to below the detection limit at 24 h. Hydrogen peroxide concentrations were also markedly lower than the carbonyl concentrations.

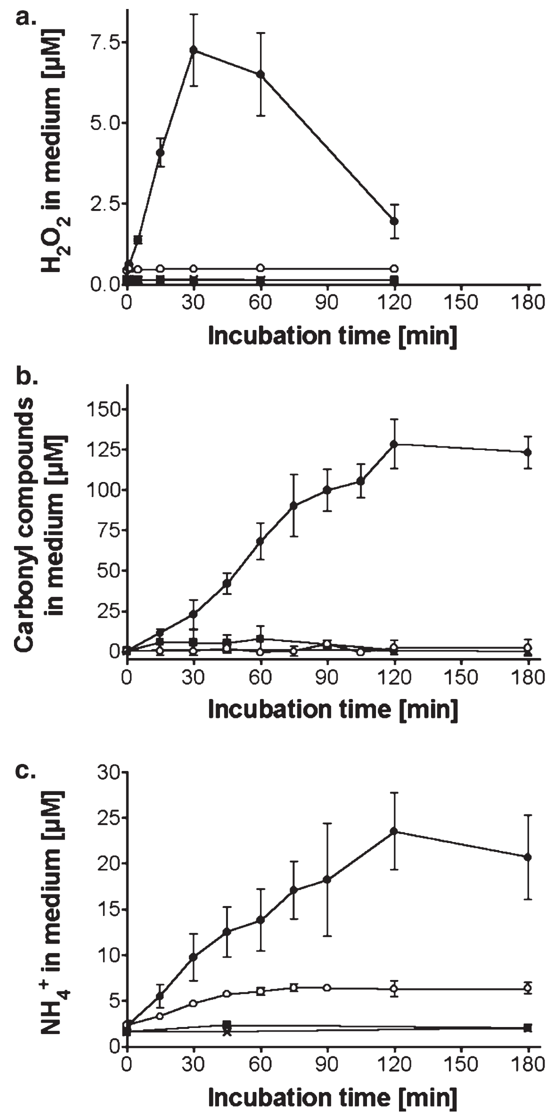

Fig. 1. Kinetics of the accumulation of H_2O_2 (a), carbonyl compounds (b) and NH_4^+ (c) in the incubation medium of *C. crispus* wild-type gametophytes from France upon exposure to L-Asn. The parameters were quantified in sea water (crosses), in sea water with addition of 168 μM L-Asn (filled squares), in sea water with *C. crispus* (open circles), and in sea water with *C. crispus* and addition of L-Asn (filled circles). Bars indicate standard deviation; *n*=3.

Supply of fresh L-Asn resulted in an immediate generation of more H_2O_2, carbonyl compounds and NH_4^+. By contrast, the production of these metabolites immediately stopped when *C. crispus* was transferred into L-Asn-free

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medium (data not shown). During repeated cycles of exposure and non-exposure to L-Asn, the H_2O_2 release was slightly reduced from the second cycle onwards (Fig. 2A), which was correlated with an increased capability of *C. crispus* to scavenge H_2O_2 (Fig. 2B).

The necessary doses of L-Asn for induction of half-maximal H_2O_2 release proved to be similar in *C. crispus* gametophytes and tetrasporophytes originating from both sides of the Atlantic (13.2–26.1 μM L-Asn; Table 1) and the same was true for the maximal responses that were observed (Table 1). Similar Michaelis constants were also computed for O_2 consumption and release of carbonyl compounds and NH_4^+ in gametophytes (14.3–40.6 μM L-Asn; Table 1), and tetrasporophytes showed comparable responses when they were exposed to equal concentrations of L-Asn (data not shown). Addition of L-Asn to cell wall macerate of *C. crispus* gametophytes, prepared with cellulase and carrageenase, resulted within 45 min in a production of carbonyl compounds. Comparable treatment of *C. crispus* protoplasts—purified from the cell wall macerate and tested intact as well as after osmolysis in distilled water—did not cause such an effect (Fig. 3).

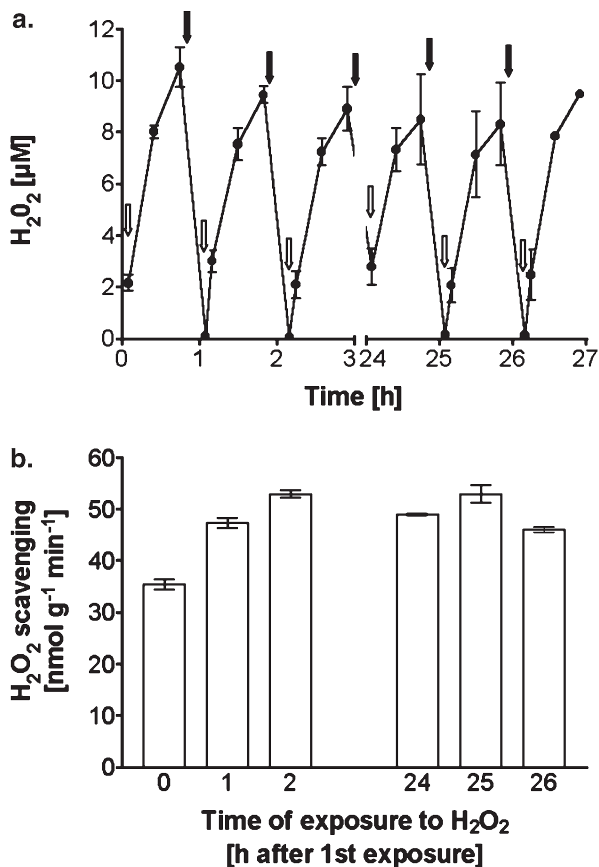

Fig. 2. (a) Development of H_2O_2 concentrations in the incubation medium of *C. crispus* wild-type gametophytes from France during six cycles of addition and removal of L-Asn. Bars indicate the standard deviation; *n*=3. White arrows indicate addition of 168 μM L-Asn to the medium; black arrows indicate replacement of the medium with fresh, asparagine-free medium. (b) Scavenging of H_2O_2 by *C. crispus* wild-type gametophytes from France within 5 min of exposure to 15.9±1.3 μM H_2O_2. Bars indicate standard deviation; *n*=3. As in (a), the same algae were retested 1, 2, 24, 25, and 26 h after the first exposure.

Uptake of L-Asn by C. crispus

As shown by uptake analysis of externally administered [^{14}C]L-Asn, *C. crispus* gametophytes sequestered L-Asn from the medium for only 10 min after the addition of the amino acid (Fig. 4). The amount that was taken up was low in comparison to the amount of L-Asn that was broken down under identical conditions (Fig. 1B). Moreover, most L-Asn that had accumulated in *C. crispus* was released by five subsequent washings of 5 s in L-Asn-free sea water. During 1 h of incubation in 168 μM L-Asn, only 0.18±0.09 nmol g^{-1} min^{-1} were taken up by the algal biomass.

Specificity and pharmacological characterization of the metabolization of L-Asn

Neither *N*-α-acetyl-L-Asn nor succinamic acid induced significant responses in *C. crispus* gametophytes at concentrations up to 1 mM. Of the 24 amino acids that were tested, only L-phenylalanine (L-Phe), L-lysine (L-Lys), and L-methionine (L-Met) induced a significant release of H_2O_2, although not as strong as L-Asn (Fig. 5). These three amino acids also induced a release of carbonyl compounds and—with the exception of L-Met which interferes with the assay used—of NH_4^+ (data not shown).

Potassium cyanide (target: metalloenzymes and amino acid oxidase), quinacrine (target: flavin-dependent redox enzymes, in particular, oxidases), and chlorpromazine (target: amino acid oxidase and various other enzymes) completely inhibited the oxidative response of *C. crispus* after exposure to L-Asn (Table 2). By contrast, the inhibitors of NADH- and NADPH-dependent enzymes, diphenylene-iodonium and imidazole, did not affect the oxidative burst response.

Ammonium chloride (Table 2), but not potassium chloride or calcium chloride, inhibited the response at millimolar concentrations, while Kryptofix 222, an NH_4^+-chelating agent, increased the response when it was added in the medium at concentrations above 100 μM. Increases by 87% and 185% were observed after application of 1 mM and 6 mM Kryptofix 222, respectively. No inhibition was observed after application of the peroxidase-inhibitor salicylhydroxamic acid. Various compounds which are known to modify the permeability of the plasma membrane for protons (2,4-dinitrophenol), K^+ (4-aminopyridine, valinomycin), Ca^{2+} (verapamil, A23187), anions (anthracene-9-carboxylic acid), or amino acids [(diethylpyrocarbonate (DEPC)], as well as the protein-kinase inhibitor staurosporine, also did not affect the H_2O_2 release response of the

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Table 1. *Kinetic constants of the responses of* C. crispus *to incubation with* L*-asparagine*

Response	Material tested	Maximal response[a]	K_m[b]
Release of H_2O_2	Gametophyte wild-type France	2.4 (2.2–2.7)	13.2[c]
	Sporophyte wild-type France	2.0 (1.7–2.4)	17.8 (2.3–33.5)
	Gametophyte wild-type Canada	2.2 (2.0–2.5)	13.9 (5.3–22.5)
	Sporophyte wild-type Canada	2.4 (2.2–2.6)	26.1 (16.5–35.8)
O_2 consumption	Gametophyte wild-type France	15.3 (11.9–18.7)	14.3 (4.9–23.7)
Release of carbonyl compounds	Gametophyte wild-type France	41.7 (35.1–48.3)	40.6 (11.5–69.7)
Release of NH_4^+	Gametophyte wild-type France	20.0 (16.8–23.1)	27.7 (12.3–43.1)

[a] Maximal H_2O_2 release response [nmol g^{-1} min^{-1}] and
[b] the necessary asparagine concentration [μM] for half-maximal response induction with L-asparagine are given. Brackets indicate 95% confidence intervals.
[c] Data taken from Weinberger *et al.* (2002).

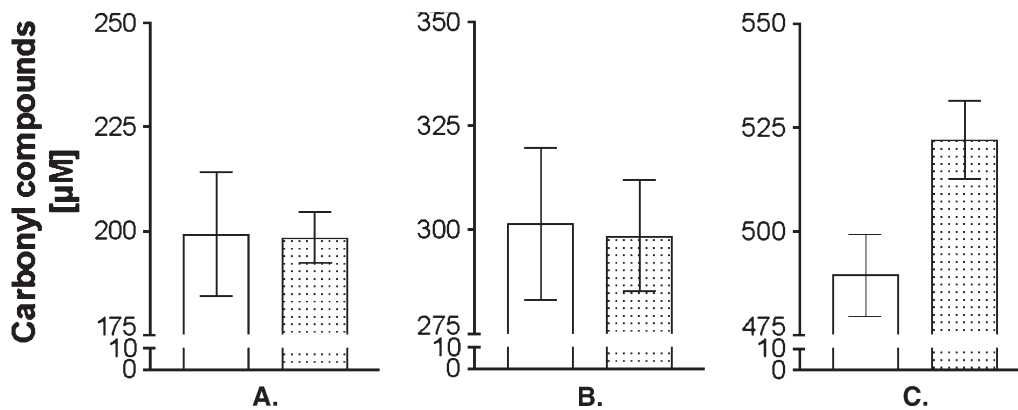

Fig. 3. Concentrations of carbonyl compounds in the medium of protoplasts prepared from *C. crispus* wild-type gametophytes from France before (A) and after (B) osmolysis in distilled water and in *C. crispus* cell-wall macerate (C). The measurements were conducted 0 min (white columns) and 45 min (stippled columns) after addition of 168 μM L-Asn. Bars indicate standard deviation; *n*=3.

gametophytes following exposure to L-Asn (data not shown).

Accumulation of H_2O_2 following exposure of C. crispus *tetrasporophytes to L-Asn controls the algal epiflora*

The survival of *C. crispus* tetrasporophytes and of *A. operculata* zoospores in the presence of H_2O_2 was monitored by staining with Evans blue. As indicated by their capacity to exclude the dye, filaments of *A. operculata* proved to be more sensitive to H_2O_2 than *C. crispus* tissue (Fig. 6). Some cells of the green algal endophyte were already affected at H_2O_2-concentrations of 100 μM, whereas *C. crispus* was unaffected by concentrations as high as 5 mM. About 1 mM of H_2O_2 was necessary for a half-maximal damage of the endophyte tissue, a concentration approximately 20 times lower than the EC_{50} for the red algal tissue (19.4 mM).

As indicated by the numbers of *A. operculata* zoospores that settled and developed within 1 week on the walls of Petri dishes following repeated exposure to 168 μM L-Asn (Table 3), the survival of the endophyte spores was not directly affected by the amino acid. By contrast, repeated exposure to L-Asn reduced by 86% the number of spores that settled on *C. crispus* tetrasporophyte JC001PC-S. Suppression of zoospore settlement was prevented by the addition of catalase to the medium, together with L-Asn. Further, a single addition of 168 μM L-Asn to *C. crispus* wild-type gametophytes was sufficient to eliminate, within 30 min, 57% of the associated bacteria (from 12.0 ± 10^4 to 5.1 ± 10^4 colony forming units g^{-1}; *n*=3).

Discussion

Chondrus crispus *features an apoplastic* L*-amino acid oxidase specific for L-Asn*

It is shown here that the red alga *C. crispus* readily breaks down L-Asn into H_2O_2, NH_4^+, and a carbonyl compound (Fig. 1), a process which is accompanied by the consumption of oxygen (Table 1). In a previous report, LC-MS analyses showed that the carbonyl compound released by *C. crispus* after exposure to L-Asn is succinamic acid (Weinberger *et al.*, 2002).

The response of *C. crispus* to incubation in the presence of L-Asn was inhibited by relatively few pharmacological agents. Compounds like DPI (Bouarab *et al.*, 1999) or anthracene-9-carboxylic acid, A23187, and staurosporine (K Bouarab, unpublished observations) clearly affect the

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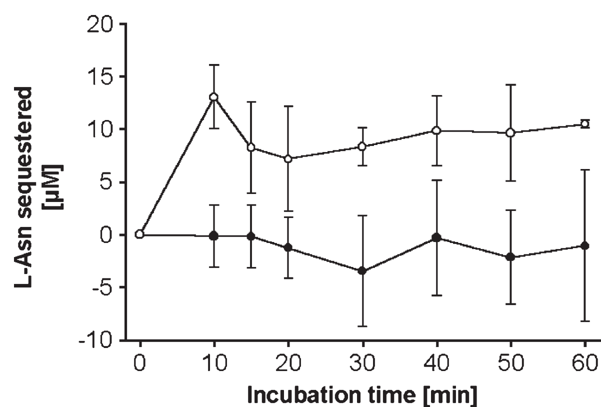

Fig. 4. Uptake of L-Asn from sea water in Petri dishes with (open circles) and without (filled circles) *C. crispus* wild-type gametophytes from France. The initial L-Asn concentration was 168 µM. Bars indicate standard deviation; *n*=3.

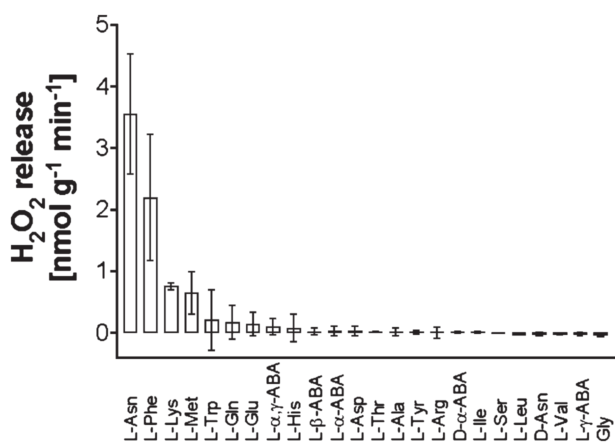

Fig. 5. Release of H_2O_2 by *C. crispus* wild-type gametophytes from France after exposure to 168 µM concentrations of various amino acids. ABA, Amino butyric acid. Bars indicate standard deviation; *n*=3.

oxidative burst in *C. crispus* thalli in response to defence elicitation. Nonetheless, they were without effect in the present study, which indicates that NADPH-oxidase and signalling pathways involving protein phosphorylation or Ca^{2+} translocation were probably not activated by L-Asn. Moreover, plant receptor stimulation usually results in a refractory state of several hours, during which no new response is possible (Navazio *et al.*, 2002). Such a refractory state was not observed in the present study; release of H_2O_2 ceased when L-Asn was removed or used up and resumed again directly after application of fresh L-Asn (Fig. 2). L-Asn, therefore, probably does not act as a signal specific for a receptor in *C. crispus*. The strict dependence of H_2O_2 release on the presence of L-Asn instead points to a role for L-Asn as a substrate for an enzyme.

The pharmacological agents that inhibited the response of *C. crispus* to L-Asn were quinacrine, chlorpromazine, and potassium cyanide (Table 2). All three are relatively unspecific, but are known to inhibit, among other enzymes, L-amino acid oxidase (Fujisawa *et al.*, 1982; Pistorius, 1983; Zollner, 1990; Vallon *et al.*, 1993). The presence of high concentrations of NH_4^+ also inhibited the reaction (Table 2), while it was activated after trapping of NH_4^+ with the chelating agent Kryptofix 222.

Table 2. *Inhibition constants for different agents that completely inhibit the oxidative burst response of* C. crispus *wild-type gametophytes from France to 168 µM L-Asn*

The necessary concentration for half-maximal inhibition is given. Brackets indicate 95% confidence intervals.

Agent	EC_{50}
Chlorpromazine	11.8 µM (8.1–17.1)
Quinacrine	43.4 µM (29.3–64.3)
KCN	601 µM (305–1186)
NH_4Cl	42.6 mM (27.0–67.0)

No response was observed with D-Asn (Weinberger *et al.*, 2002) and most proteinogenous amino acids (Fig. 5). N-modified derivatives of L-Asn were also not accepted as a substrate, indicating the involvement of a stereospecific reaction with an absolute requirement for the presence of a free amino group. Based on the release of H_2O_2, L-Phe, L-Lys, and L-Met were also metabolized by *C. crispus*, yet at much lower rates than L-Asn (Fig. 5).

Altogether, these observations indicate that *C. crispus* features an L-amino acid oxidase, which specifically metabolizes L-Asn as follows:

$$\text{L-Asn} + O_2 + H_2O \rightarrow \text{2-oxo-succinamic acid} + NH_3 + H_2O_2$$

L-Amino acid oxidases (EC 1.4.3.2) are flavoenzymes which catalyse the oxidative transformation of L-amino acids to 2-imino-acids, which are subsequently hydrolysed into 2-keto-acids and ammonia. Two electrons are transferred from the amino acid to the flavin cofactor, which then reduces molecular oxygen to H_2O_2 (Curti *et al.*, 1992). Consistent with this mechanism, the amounts of L-Asn necessary for the induction of half-maximal responses in oxygen consumption and the release of H_2O_2, carbonyl compounds, and NH_4^+ were of the same order of magnitude (Table 2). That these compounds were not observed in 1:1 stoichiometric ratios (Fig. 1) is probably due to concomitant scavenging of H_2O_2 (Fig. 2B) and uptake of NH_4^+ by the red alga.

An L-Asn-dependent release of H_2O_2, NH_4^+, and 2-oxo-succinamic acid could involve transport of L-Asn into the cell followed by an intracellular breakdown of the amino acid and release of the products (Palenik and Morel, 1990*a*). Transport proteins, however, were apparently not necessary for the oxidation of L-Asn by *C. crispus*, since the response proved to be fully insensitive to the ionophores 2,4-dinitrophenol and valinomycin, as well as to DEPC. In addition, uptake of labelled L-Asn from the incubation medium was very limited (Fig. 4) and the label was readily washed out from the algal biomass, showing

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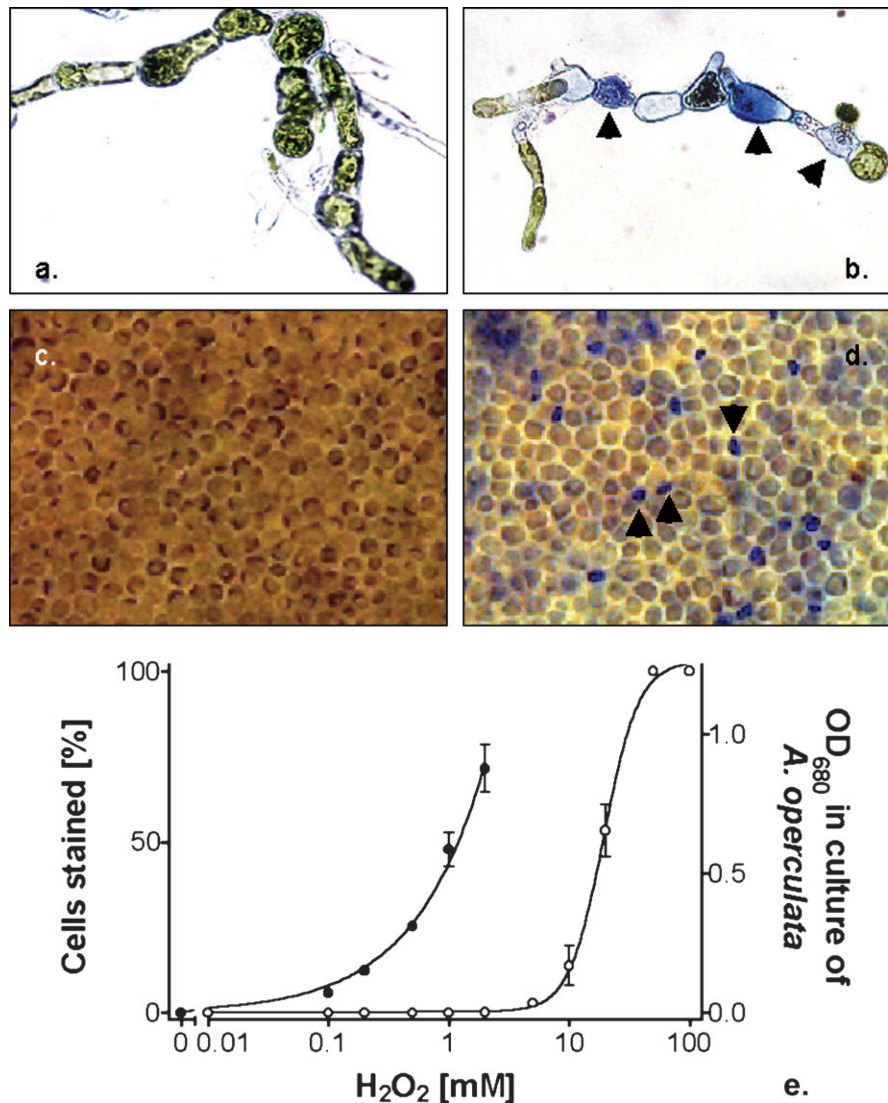

Fig. 6. Sensitivity of *A. operculata* and *C. crispus* wild-type gametophytes from France to H_2O_2. Cells of *A. operculata* (a, b) and *C. crispus* (c, d) after treatment without H_2O_2 (a, c), with 1 mM H_2O_2 (b) or with 10 mM H_2O_2 (d) and subsequent staining with Evans blue. The diameters of *A. operculata* and *C. crispus* cells were approximately 15 μM and 5 μM, respectively. Arrows indicate some of the cells that were stained. (E) Dose–response curves of H_2O_2 toxicity to *A. operculata* (filled circles, quantified as optical density at 680 nm) and *C. crispus* (open circles, quantified as relative amount of stained cells). Bars indicate standard deviation; *n*=3. Lines represent best fitting logistic functions.

that it was rather adsorbed in the algal free space than taken up. The L-amino acid oxidase is therefore probably an apoplastic enzyme. This is further supported by the fact that cell wall macerate of *C. crispus* generated carbonyl compounds from L-Asn. Intact protoplasts—devoid of the cell wall—were inactive, and osmolysis of these protoplasts resulted in a release of cytoplasmic carbonyl compounds, but not in additional production of such compounds from L-Asn (Fig. 3). Corresponding with these observations, protein extracts of *C. crispus* never expressed L-amino acid oxidase-like activity, while extraction pellets—presumably containing cell wall debris—generated carbonyl compounds from L-Asn (data not shown).

Among eukaryotic photosynthetic organisms, L-amino-acid oxidases have so far been reported from two red macroalgae, *Gymnogongrus flabelliformis* (Fujisawa *et al.*, 1982) and *Amphiroa crassissima* (Ito *et al.*, 1987), as well as from the unicellular algae *Chlamydomonas reinhardtii* (Muñoz-Blanco *et al.*, 1990; Piedras *et al.*, 1992; Vallon *et al.*, 1993), *Pleurochrysis* spp. (Palenik and Morel, 1990*a*), *Prymnesium* spp. (Palenik and Morel, 1990*b*), and *Amphidinium* spp. (Palenik and Morel, 1990*b*). The L-amino acid oxidase of *C. crispus* shows similarities with that of *C. reinhardtii*. Both enzymes are extracellular (Muñoz-Blanco *et al.*, 1990) and are inhibited by NH_4^+ (Muñoz-Blanco *et al.*, 1990; Vallon *et al.*, 1993). By contrast, the L-amino acid oxidase

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Table 3. *Settlement of* A. operculata *zoospores on the tetrasporophytic* C. crispus *strain JC001PC-S and on polystyrene Petri dishes*

The settlement was monitored 5 d after inoculation, with and without addition of 1100 U ml^{-1} catalase and 168 μM L-Asn to the medium. + and − refer to presence and absence, respectively.

	Catalase	L-Asn	Settled spores mm^{-1}
Petri dish	−	−	41.0±9.9
	−	+	41.6±20.1
C. crispus	−	−	9.9±4.4
	−	+	1.4±1.2
	+	+	8.7±1.7

of *G. flabelliformis* is intracellular and insensitive to NH_4^+ (Fujisawa *et al.*, 1982).

Biological significance of C. crispus *L-amino acid oxidase*

Release of nitrogen from amino acids has until now been regarded as the main ecophysiological role of L-amino acid oxidases in algae. The extracellular L-amino acid oxidases detected in planktonic organisms are induced under nitrogen-depleted conditions, metabolize a wide spectrum of free amino acids, and allow for growth with amino acids as the sole nitrogen source (Muñoz-Blanco *et al.*, 1990; Palenik and Morel, 1990*a*, *b*). *Chondrus crispus* would also be capable of using nitrogen from free amino acids for growth, since *c*. 50% of the NH_4^+ released from L-Asn was sequestered (Table 2). However, the total concentration of free amino acids in coastal sea water does not exceed 1 μM, and L-Asn, L-Phe, L-Lys, and L-Met together usually account for not more than 10% of this amount (Chau and Riley, 1966; Clark *et al.*, 1972; Jørgensen, 1982). Not more than 0.1 μM of free amino acids are therefore available in free sea water for oxidation by *C. crispus*. Nonetheless, oxidation of L-Asn by *C. crispus* was detected only at concentrations above 1 μM (Weinberger *et al.*, 2002), and the necessary concentrations for half-maximal activity were at least 10 times higher (Table 1). These kinetic constants are at least 100 times too high to allow for an efficient uptake of amino acid-bound nitrogen from sea water.

Up to 100 μM Asn are present in the medium of *A. operculata* cultures (50 g biomass l^{-1}), and this concentration more than doubles when κ-carrageenan oligosaccharides are present in the culture medium and accelerate the excretion (Weinberger *et al.*, 2002). Further, *A. operculata* is endowed with the enzymatic equipment to degrade κ-carrageenans (Bouarab *et al.*, 1999). It is thus assumed that after the invasion of *C. crispus*, *A. operculata* filaments excrete L-Asn, a process further enhanced upon recognition of κ-oligocarrageenans, when the endophyte reaches the inner cortex of gametophytic hosts. It follows that L-Asn would accumulate in the host apoplasm surrounding the pathogen, and local concentrations significantly higher than 100 μM would result in the cortex. In this situation, *C. crispus* L-amino acid oxidase should enable the host to gain nitrogen from its attacker.

The build-up of L-Asn concentrations at the site of attack would also result in a steady release of H_2O_2, and the question arises are the concentrations reached sufficient to kill the pathogen. No local quantification of H_2O_2 at the penetrating sites was possible, but indirect evidence is available indicating that sufficient concentrations of this active oxygen species are locally present; addition of L-Asn to a tetrasporophytic strain inoculated with *A. operculata* zoospores reduced the settlement of the endophyte by 86% (Table 3). This effect was prevented by simultaneous addition of catalase, an enzyme transforming H_2O_2 into molecular oxygen and water, which indicates that oxidation of the amino acid is an essential part of the protective action of L-Asn.

Another indication for the build-up of elevated amounts of H_2O_2 in the apoplast of *C. crispus* can be obtained by monitoring its bacterial flora. In the present study, exposure of *C. crispus* gametophytes to L-Asn resulted in the elimination of 57% of the bacterial flora within 30 min, suggesting that the oxidation of the amino acid locally leads to H_2O_2 concentrations high enough to kill associated bacteria. Certain bacteria associated with seaweeds are relatively sensitive to H_2O_2. Concentrations in the range of 20–600 μM were sufficient for the elimination of 50% of the bacterial flora associated with *Gracilaria conferta* (Weinberger and Friedlander, 2000) or *Laminaria digitata* (Küpper *et al.*, 2001). *Acrochaete operculata* filaments proved to be comparatively sensitive to H_2O_2 as the concentration that damaged half of the endophyte filament cells (*c*. 1 mM) was markedly lower than that required to affect half of *C. crispus* gametophytic cells (*c*. 20 mM) (Fig. 6). In the interaction between tomato and its fungal pathogen *Colletotrichum coccodes*, H_2O_2 generated extracellularly by the host prevents penetration of the pathogen, either by its cytotoxicity or by its involvement with cell-wall cross-linking (Mellersh *et al.*, 2002). Similarly it is proposed that oxidation of L-Asn contributes to the resistance of *C. crispus* gametophytes against *A. operculata* by hindering zoospore settlement and containing filament growth within the host tissue.

Altogether nitrogen sequestration is probably not the primary role of the L-amino acid oxidase of *C. crispus*. This enzyme is more likely to be involved with the control of the infection by its endophyte. Another possible target of this mechanism could be members of the genus *Ulva*, a widely distributed epiphytic pest. These contain large amounts of free L-Asn (Kakinuma *et al.*, 2001) and are known to excrete amino acids (Jørgensen, 1982).

The present study further confirms the important role of host cell wall carrageenans for the interaction of *C. crispus* with *A. operculata*. Only host tetrasporophytes contain κ-carrageenan, which is required to activate the secretion of L-Asn by the pathogen, which in turn provides the substrate

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for H_2O_2 generation. In addition to L-amino acid oxidase, *C. crispus* probably has further means to control *A. operculata*. Cell-free extracts of *A. operculata* have been shown to elicit a DPI-sensitive oxidative burst response in *C. crispus* gametophytes, which results in a modification of protein expression patterns and of resistance to *A. operculata* (Bouarab *et al.*, 1999, 2004). Attempts to identify this signal in *A. operculata* cell-free extracts are currently under way in the authors' laboratory.

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