Physiological and Molecular Plant Pathology 75 (2010) 38–45

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

# Sulfated macroalgal polysaccharides λ-carrageenan and ι-carrageenan differentially alter *Arabidopsis thaliana* resistance to *Sclerotinia sclerotiorum*

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ARTICLE INFO

*Article history:*
Accepted 18 August 2010

*Keywords:*
Elicitor
carrageenans
*Sclerotinia sclerotiorum*
Oxalic acid
Virulence factor
Arabidopsis
Salicylic acid
Jasmonic acid
Induced resistance
Glucosinolate

ABSTRACT

We examined the effect of macroalgal polysaccharides ι- and λ-carrageenan, which differ in sulfation, on Arabidopsis resistance to the necrotropic fungal pathogen *Sclerotinia scleortiorum*. Pretreatment of Arabidopsis with highly sulfated λ-carrageenan induced resistance to *S. sclerotiorum* resulting in smaller leaf lesions; by contrast, the less sulfated ι-carrageenan enhanced susceptibility. λ-carrageenan induced resistance correlated with increased expression of jasmonic acid related genes *AOS*, *PDF1.2* and *PR3*. Further, λ-carrageenan increased the oxalase oxidase activity *in planta*. The λ-carrageenan induced resistance in the salicylic acid deficient mutant *ics1*, but did not rescue the susceptibility of *jar1* plants suggesting that λ-carrageenan induced resistance is independent of salicylic acid. The difference in the bioactivity of carrageenans is due, at least partly, to differences in the degree of sulfation.


## 1. Introduction

Plants are exposed to a number of pathogenic microbes, some of which are highly destructive, posing a threat to plant growth and development. However, plants possess constitutive and inducible defense mechanisms to resist pathogen attack [1]. Inducible plant defense responses are activated by various elicitors that are perceived by the host; this perception results in expression of defense mechanisms against the pathogen [2]. Elicitor induced plant responses have become an important tool in the analysis of plant resistance mechanisms against pathogens [3,4].

The elicitor induced host response involves direct and/or indirect activation of complex signaling cascades [3] usually beginning with an influx of calcium and an oxidative burst [5], followed by the synthesis of defense signals such as salicylic acid, jasmonic acid and ethylene [6,7]. The induced defense is further complemented with reinforcement of cell walls by cross-linking and lignification, accumulation of anti-microbial compounds including phytoalexins and chitinase, and synthesis of anti-microbial proteins including pathogenesis-related proteins [1,5,8]. Induced plant defenses thus involve coordinated molecular [9], biochemical [2,10] and physiological responses [11].

A wide variety of molecules including oligo- and polysaccharides, peptides, proteins and lipids are potent elicitors of plant defense [12]. Seaweeds (marine macroalgae) are plant biostimulants rich in unique polysaccharides not found in land plants [13,14]. Algal products are used in agriculture to improve seed germination, plant growth and yield, and resistance against a number of abiotic and biotic stresses [15–18]. Polysaccharides, laminarin and fucans of brown algae and carrageenans of red algae have been reported to induce disease resistance in plants [4,19,20]. Carrageenans are linear units of D-galactose residues linked with alternating α-(1,3) and ß-(1,4) linkages that are substituted by one (k-carrageenan), two (ι-carrageenan), or three (λ-carrageenan) ester-sulfonic groups per digalactose unit [21,22]. The biological effects of the degree of sulfation of these polysaccharides are not clearly understood.

*Sclerotinia sclerotiorum* is a filamentous ascomycete fungus that infects more than 400 species of plants including sunflower, canola, soybean and Arabidopsis [23]. It is estimated that the pathogen can inflict as high as 100% yield losses on susceptible crop plants [24,25]. Oxalic acid secreted by the fungus is an important virulence factor required for its pathogenicity [26,27]. Oxalic acid abets pathogenesis by lowering the extracellular pH to enhance the

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doi:10.1016/j.pmpp.2010.08.003

destructive activity of fungal hydrolytic enzymes. Further, oxalic acid also suppresses host defense responses [28,29]. Genetic resistance to *S. sclerotiorum* is limited in commercial germplasm; therefore the management of this disease largely relies on disease avoidance rather than physiological resistance [30].

*Arabidopsis* has been used as a host model to study interactions with *S. sclerotiorum* [31]. Polysaccharide carrageenans have been shown to induce plant defenses to various stresses [4]; however their role in plant resistance to *S. sclerotiorum* is not known. Here we investigate the differential elicitor activity of two carrageenans (ι- and λ-) that differ in degree of sulfation and elucidated the molecular mechanisms of the defense response elicited in *Arabidopsis thaliana* ecotype Col-0.

## 2. Materials and methods

### 2.1. Plant material, culture of S. sclerotiorum, and carrageenans

Wild-type Arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] ecotype Columbia (Col-0) seed was purchased from Lehle Seeds (Round-rock, TX, USA). Arabidopsis mutants (*ics1* and *jar1*) were obtained from Arabidopsis Biological Resource Center (ABRC, Ohio State University Columbus, OH, USA). The seeds were planted in sterile peat pellets (Jiffy Co., Shippegan, New Brunswick, Canada) arranged on flat trays. The trays were kept in a plant growth room at $22 \pm 2$ °C with a photoperiod of 16 h light at 125 μmol $m^{-2}$ $s^{-1}$ and 8 h dark cycle. Three-week-old plants were used in the experiments.

The fungus *S. sclerotiorum* (Lib.) de Bary was isolated from a naturally infected stem of a sunflower (*Helianthus annuus* L.) plant. The sclerotium was surface sterilized with 2% sodium hypochlorite for 2 min and rinsed with sterile distilled water then cultured on potato dextrose agar (PDA, Difco) for 48 h in the dark at room temperature. The fungal culture was purified by sub-culturing the advancing edge of the growing mycelium on fresh PDA plates at 22 °C in the dark. Virulence of the pathogen was tested by measuring its ability to produce oxalic acid in vitro.

### 2.2. Test solution used

Two types of carrageenans (iota-(ι-), and λ-carrageenan) provided by Cargill Texturant Systems, Baupte, France were used in this study. The λ-carrageenan is highly sulfated (35%), whereas ι-carrageenan is less sulfated (30%). The spray solution was prepared using 1.0 g $l^{-1}$ carrageenan and 0.02% (v/v) of Tween-20 in sterile ultra-pure water (MilliQ).

### 2.3. Plant inoculation with S. sclerotiorum

Three-week-old Arabidopsis plants with fully expanded leaves were sprayed until runoff with 2 ml of each test solution, followed by a subsequent spray after a 5 d interval. The control plants were sprayed with sterile ultra-pure water with Tween-20 (0.02%, v/v). The plants were inoculated with *S. sclerotiorum* 48 h after the second spray. Briefly, a mycelial plug (5 mm in diameter, 5–6 mm thickness) from the advancing edge of the growing mycelium on cultured plates was removed with a cork borer and carefully placed on the adaxial surface of Arabidopsis leaves previously sprayed with test solutions. Three leaves per plant and 15 plants for each treatment were inoculated. In parallel, control plants were mock inoculated with PDA plugs. Inoculated plants were incubated in a clear plastic box covered with plastic wrap to maintain humidity at $22 \pm 2$ °C and incubated with a photoperiod of 16 h light and 8 h dark. The lesion size was measured with a digital vernier caliper once every 24 h for three days. Leaf samples for enzyme assays and nucleic acid extraction were collected at various time points and leaf tissue was sampled 1 cm around the point of inoculation using a cork borer.

### 2.4. Effect of carrageenan treatment on plant sensitivity to oxalic acid

We determined the effect of carrageenan treatment on leaf sensitivity to oxalic acid [27]. Plant material, growth conditions and carrageenan treatments were the same as described above in 'Plant Inoculation with *S. sclerotiorum*'. Two concentrations (10 and 25 mM) of OA (2 μl) were spotted on treated and control leaves. The treated plants were placed in a growth room at $22 \pm 2$ °C. The diameter of the lesion was measured on 10 individual plants from each treatment using a digital vernier caliper. The experiment was repeated twice.

### 2.5. In vitro and in planta measurement of oxalic acid

The oxalic acid (OA) concentration in *S. sclerotiorum* inoculated leaves and in potato broth was determined colorimetrically according to [32]. For *in vivo* OA determination, approximately 50 mg fresh weight (FW) of leaf tissue collected from each of the three independent plants at 24 and 48 h after inoculation was homogenized in 300 μl of phosphate buffer (100 mM, pH 6.8) and centrifuged at 10,000 rpm for 10 min. The supernatant was used for OA determination. The experiment was repeated twice with similar results. To determine OA *in vitro*, both ι- and λ- carrageenan (1 g $l^{-1}$) were added to the potato dextrose broth and inoculated with a 5 mm plug of mycelium. The inoculated broth was incubated in the dark for 72 h. The culture medium at 24, 48 and 72 h after inoculation was filtered and the filtrate was used for OA determination. Fungal mycelium was oven dried for 48 h and weighed to quantify fungus growth.

The reaction mixture for OA determination consisted of 200 μl of leaf extract or filtered culture broth, 110 μl of bromophenol blue (1 mM), 198 μl of sulphuric acid (1 M), 176 μl of potassium dichromate (100 mM) and 4.8 ml of distilled water. The reaction mixture was incubated in a water bath at 60 °C for 10 min and quenched by adding 500 μl of 0.75 M sodium hydroxide. The absorbance was read at 600 nm ($A_{600}$) with a spectrophotometer. The concentration of OA was calculated using the standard graph made with oxalic acid (Sigma–Aldrich). The quantity of OA was expressed as μg $mg^{-1}$ oxalic acid in fresh weight (fresh wt) of leaf samples or dry weight (DW) of mycelium. The assay was performed twice with similar results shown as mean of two independent experiments.

### 2.6. Oxalate oxidase assay

Oxalase oxidase (OXO) activity in the plant tissue correlates with resistance to infection by *S. sclerotiorum* [33]. We followed the method described by [34], with minor modifications, to determine the effect of carrageenan treatments on OXO activity in the leaves of treated plants. About 50 mg of leaf tissue from each replicate plant was ground in 300 μl of buffer (18 mg oxalic acid in 100 ml of 2.5 mM succinic acid, pH 4) and the reaction was incubated at 37 °C for 30 min. Next, 200 μl of developing solution [6 mg of amino-antipyrene dissolved in 30 μl of *N*,*N*-dimethylaniline, and added to 100 ml of 100 mM sodium phosphate buffer (pH 7.0) that contained 57 μl of horseradish peroxidase (Sigma–Aldrich, 140 mg $ml^{-1}$)] was added. The mixture was incubated at room temperature for 60 min and the absorbance measured at 550 nm ($A_{550}$) on a spectrophotometer.