Eur J Plant Pathol (2011) 131:237–248
DOI 10.1007/s10658-011-9802-6

Extracts of the marine brown macroalga, *Ascophyllum nodosum*, induce jasmonic acid dependent systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* DC3000 and *Sclerotinia sclerotiorum*

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Accepted: 28 April 2011 / Published online: 21 May 2011

Abstract We studied the mechanism of *Ascophyllum nodosum* (a brown macroalga) induced resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* DC3000. Root treatment of *A. thaliana* Col-0 plants with extracts of *A. nodosum* [aqueous (ANE), chloroform (C-ANE) and ethylacetate fractions, (E-ANE)] reduced the development of disease symptoms on the leaves. These extracts also induced resistance in salicylic acid deficient NahG and *ics1* plants. However, the extracts did not elicit an effect on *jar1* (jasmonic acid resistance 1) mutant. *A. nodosum* extract induced resistance to *Pst* DC3000 correlated with increased expression of jasmonic acid related gene transcripts *PDF1.2* while *PR1* and *ICS1* expression were less affected. Additionally, pretreatment of Arabidopsis plants with ANE, protected the plants from a necrotroph, *Sclerotinia sclerotiorum*. The results suggest that the *A. nodosum* extracts can induce resistance in Arabidopsis to different pathogens which is largely jasmonic acid dependent.

Keywords *Ascophyllum nodosum* · Induced systemic resistance · JA dependent defence response · *Pseudomonas syringae* pv. *tomato* DC3000

Electronic supplementary material The online version of this article (doi:10.1007/s10658-011-9802-6) contains supplementary material, which is available to authorized users.

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Introduction

Plants encounter various biotic and abiotic stresses during life cycle that limit their growth and productivity. Among biotic stresses, microbial pathogens are highly destructive that could lead to death of the plant. Plants respond to microbial pathogens through activation of genes and biochemical pathways to protect against the invading organisms. Such response is induced by chemicals commonly referred to as elicitors. Elicitors are of diverse chemical origin like peptides, carbohydrates and lipids. Research in this area suggests this strategy could be successfully used to protect plants against microbial pathogens.

Plants employ various signalling pathways such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) to respond to pathogen attack (Grant and Lamb 2006; Howe 2004). SA, JA, and ET acting either individually or in combination are involved in both basal and induced resistance in plants against various pathogens (van Pieterse et al. 1998). Two types of induced disease resistance mechanisms are recognized in plants—systemic acquired resistance (SAR) and induced systemic resistance (ISR). In SAR, preinfection of plants with a necrotizing pathogen leads to an enhanced resistance to further infection in distal plant parts (Ryals et al. 1996). The systemic acquired resistance is associated with an increase of *in planta* SA, with the resistance being broad spectrum. SA mediated systemic resistance is also associated with the expression of pathogenesis related (PR) genes (Gaffney et al. 1993; Van Loon 1997). However, SA-independent disease resistance mechanisms are also reported which are equally effective against pathogens (van Pieterse et al. 1996). One such mechanism is ISR which is triggered by non-pathogenic microbes and is largely associated with JA and ET dependent mechanisms (van Pieterse et al. 1998; Thomma et al. 2001). Typically, SAR markers are *PR1* genes and other acidic *PR* genes, while JA mediated responses involve basic *PR* genes and other proteins such as thionins, defensins (sulphur-rich proteins) and proteinase inhibitors (Thomma et al. 2002; Zhou 1999). Both SAR and ISR offer protection against a broad spectrum of pathogens.

Plant defence mechanisms involving SA, JA and ET can also be triggered by certain abiotic stimuli i.e., natural and synthetic chemicals moieties. Chemicals that mimic SA [e.g., benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH),β–aminobutyric acid, (BABA), 2,6-dichloro-isonicotinic acid (INA)] or JA [e.g., methyl jasmonate (MeJA)] have been investigated for their ability to induce SAR and ISR against various plant pathogens and some of these chemicals are available for use in agriculture (van Pieterse et al. 1998; Zimmerli et al. 2000). Induced plant resistance, through the application of synthetic chemicals and components from plant and animals, is often viewed as a potential alternative to chemical control of plant diseases in agricultural systems (Benhamou 1996).

Seaweeds (marine macroalgae) are rich sources of nutrients and bioactive compounds. A number of brown seaweeds such as *Ecklonia maxima*, *Laminaria saccharina*, *Fucus serratus*, *F. vesiculosus, Macrocystis* spp., *Sargassum* spp. and *Asophyllum nodosum* are extensively used in agriculture (Craigie 2010). *Ascophyllum nodosum* is the most widely used seaweed in agriculture and grows along the Atlantic coastlines of North America and Europe (McLachlan 1985; Ugarte et al. 2006). A few studies show that application of seaweed extracts to plants resulted in direct or indirect protection against pathogens. For example, soil application of liquid seaweed extracts to cabbage (*Brassica oleracea* var. *capitata*) stimulated microbes that were antagonistic to *Pythium ultimum,* resulting in reduced incidence of the damping-off disease in seedlings (Dixon and Walsh 2002). Similarly, extracts of a number of seaweeds have been reported to reduce the severity of foliar diseases (Jayaraj et al. 2008; Jimenez et al. 2011).

Marine algae, in general, are rich in unique polysaccharides that can be potent elicitors of plant defence responses (Cluzet et al. 2004; Mercier et al. 2001; Sangha et al. 2010). The polysaccharide λ-carrageenan induced JA dependent response of Arabidopsis suppressed *S. sclerotiorum* (Sangha et al. 2010). Similarly, carrageenans found in certain red algae elicited defence response in tobacco against *Phytophthora parasitica* var. *nicotianae* (Ppn) inducing high levels of SA. Interestingly, laminaran, a key polysaccharide of brown algae, regardless of the concentration used, did not induce accumulation of SA (Mercier et al. 2001) indicating specificity of activity of marine polysaccharides that could induce different pathways. The brown alga, *A. nodosum* is the most widely used seaweed in agriculture and horticultural crop production (Rayorath et al. 2008). *A. nodosum* contains laminaran (β-D-(1→3) glucan) that elicits plant growth and defence responses by the induction of antimicrobial phytoalexins (Patier et al. 1993). Brown algae also contain sulphated fucans with the ability to induce plant disease resistance. However, mechanisms of *A. nodosum* extract-induced disease resistance have not been investigated. Therefore, in this study, we investigated elicitor activity of aqueous (ANE) and organic (C-ANE, E-ANE) subfractions of *A. nodosum* extract against *Pseudomonas syringae* pv. *tomato* DC3000 in *A. thaliana* Col-0. Further, using genetic and molecular tools we studied the mechanism of *A. nodosum* extract

induced plant resistance to *P. syringe* pv *tomato* DC3000 and *S. sclerotiorum*.

Materials and methods

Plant material, microbial culture and maintenance

Seeds of *Arabidopsis thaliana* Col-0 were purchased from Lehle Seeds (Round Rock, TX, USA). The transgenic line NahG that accumulates little or no salicylic acid (Delaney et al. 1994) was a kind gift from Dr. Xinnian Dong, Duke University, NC, USA. Transgenic PR1::GUS seeds were provided by Dr. Allan Shapiro and pAOS::GUS were gifted by Dr. Innes Kubigsteltig. Jasmonic acid resistant mutants, *jar-1* (Staswick et al. 1992) and *ics1* were obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University, Columbus, Ohio USA. The seeds were planted in Jiffy peat pellets (Jiffy Inc., NB, Canada), and grown in a growth chamber at 22°C±2°C with a photoperiod of 16/8 h day/night cycle. For histochemical experiments, PR1::GUS and pAOS::GUS plants were grown on solidified half-strength Murashige and Skoog Basal (MS) Medium (Sigma, Oakville, ON, Canada) supplemented with 1% sucrose.

Pseudomonas syringae pv. *tomato* DC3000 was a kind gift from Dr. Diane Cuppels, Agriculture and Agri Food Canada (AAFC), London, Ontario, Canada. The bacterium was maintained on King's medium B (10 g peptone, 1.5 g potassium phosphate monobasic, 15 g glycerol, 7 g bacteriological agar at pH 7.0. 5 ml of 1 M $MgSO_4$/l) (King et al. 1954) containing 50 μg/ml rifampicin. The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary was isolated and purified from naturally infected sunflower (*Helianthus annuus* L.) (Sangha et al. 2010) and maintained on potato dextrose agar (PDA, Difco) medium.

Preparation of *Ascophyllum nodosum* extracts

Powdered extract of *Ascophyllum nodosum* was provided by Acadian Seaplants Limited (Dartmouth, Nova Scotia, Canada). Aqueous seaweed extract (ANE) was prepared by dissolving the powder in double distilled water (1 g/l), filter-sterilized using 0.2 μm filters (Corning) and stored at 4°C until use. The organic sub-fractions were prepared using 10 g of the *A. nodosum* extract powder suspended in 40 ml methanol and shaken vigorously for 15 min (Rayorath et al. 2008). The suspension was centrifuged at 4,000 × *g* for 10 min to collect the supernatant which was evaporated under nitrogen to dryness. The resulting dried methanol extract was suspended in 10 ml of methanol to constitute the methanol extract (M-ANE) or suspended in 50 ml distilled water for sequential fractionations using chloroform and ethyl acetate to obtain the chloroform (C-ANE) and ethyl acetate (E-ANE) fractions, respectively. Briefly, 75 ml of chloroform was added to the aqueous fraction, mixed vigorously and the organic phase separated using a separating funnel. The remaining aqueous portion was extracted three times with the same volume of ethyl acetate and the organic fraction was separated. The fractions were dried under nitrogen and finally re-suspended in 10 ml methanol and stored at 4°C for further use. For treatments, the final concentration of *A. nodosum* extracts used was 1 ml/l (1 g/l equivalent) in distilled water (v:v). For a control, 1 ml methanol was added to distilled water.

Effect of *Ascophyllum nodosum* extracts treatment on disease development

Three-week-old plants (wild type Col-0, the transgenic line NahG and the mutants *jar1* and *ics1*) were irrigated with 25 ml of the ANE, C-ANE and E-ANE (1 g/l). For a positive control, 1 mM of 2, 6-dichloroisonicotinic acid (INA) was used while control plants received the same volume of sterile distilled water with 0.1% methanol. Plants were maintained in a growth chamber at a 16/8 h day/night cycle and 22°C±2°C. Fully expanded leaves in the second and third whorl were pressure inoculated with *Pst* DC3000 (0.01 OD_{600} in 10 mM $MgSO_4$ solution) 48 h after seaweed treatment using a 1 ml syringe without the needle (Katagiri et al. 2002) until one half of the lamina appeared water soaked. The inoculated plants were transferred back to the growth chamber and observed for bacterial growth and disease development. Each experiment was repeated three times with five replicates.

Five leaves per treatment were collected at 48, 72 and 96 h after inoculation, weighed and macerated immediately in a Kontes tissue grinder (Fisher

Scientific, PA, USA) using sterile distilled water. The suspension was plated on King's Medium B at various dilutions in sterile water. The plates were incubated for 48 h at 28°C and the number of colony forming units (cfu)/g fresh weight (FW) was recorded.

Plants were scored after 5 days of inoculation on 0–4 scale (0=no infection, 1=25%, 2=50%, 3=75% and 4=100%) assigned to the percentage of disease spread on the leaves. Disease severity (DI) was calculated using the formula of Singh and Prithiviraj (1997):

$$DI = \{\text{Sum of ratings } (0-4)/\text{Maximum possible score} \times \text{Total number of leaves examined}\} \times 100$$

For *Sclerotinia sclerotiorum* inoculation, three-week-old wild type Arabidopsis plants (Col-0) with fully expanded leaves were root treated with 25 ml of *A. nodosum* aqueous extract (1 g/l) and sprayed with 2 ml of the same extracts. Three leaves per plant were inoculated at 48 h after the treatment with 5 mm mycelial plug of *S. sclerotiorum* placed on leaves and the plants were maintained at 22±2°C with a photoperiod of 16 h light and 8 h dark. Ten plants for each treatment were inoculated. Size of lesions were measured once every 24 h for 4 days. The experiment was repeated twice.

The effect of *A. nodosum* extract treatment on leaf sensitivity to oxalic acid (OA), a pathogenicity factor of *S. sclerotiorum*, was also determined. Plant material, growth conditions and *A. nodosum* extract treatments were the same as described above. Two concentrations (10 and 25 mM) of OA (2 µl) were spotted on the leaves of ANE and water treated control plants. The OA spotted plants were placed in a growth room at 22±2°C. The diameter of the lesions were measured on ten individual plants from treated and control plants. The experiment was repeated twice.

In vitro growth of *Pst* DC3000 in *Ascophyllum nodosum* extracts

Solution of aqueous (ANE) and organic sub-fractions (C-ANE, E-ANE) were prepared (100, 50, 25, 12.5 and 6.25 µg/ml). *Pst* DC3000 at a concentration of 0.01 OD_{600} in King's Medium B was amended with extracts in a 96-well plate using 200 µl of culture medium and incubated at 28°C for 24 h. The bacterial growth was measured using a 96-well microplate reader (Synergy ™ HT controlled via KC4 ™ PC software) at λ 600 nm (Zhang et al. 2006). The experiment was repeated three times.

Histo-chemical analysis GUS in PR1::GUS and AOS::GUS plants

Seven-day-old seedlings (three plants per treatment) of transgenic *Arabidopsis* carrying PR1::GUS reporter and AOS::GUS grown in half strength MS medium were transferred to 12-well tissue culture plates containing 1 ml of liquid half-strength MS medium for 2 days. The MS medium was replaced with 1 ml solution of *A. nodosum* extract [ANE, C-ANE, E-ANE (1 g/l)], 1 mM INA, or control (0.1% methanol)] and placed on a gyratory shaker set at 90 rpm and a 16/8 h day/night cycle. The seedlings were stained for localizing GUS activity (Jefferson et al. 1987) 48 h after treatment.

Phenylalanine ammonia lyase estimation

The activity of phenylalanine ammonia lyase (PAL) was measured based on the amount of cinnamic acid formed with phenylalanine as the substrate following standard published protocol. Plants were treated with seaweed extracts, 1 mM INA or control and leaf samples were collected at 24, 48, 72, 96 and 120 h after treatment. The amount of cinnamic acid was calculated using a standard graph of cinnamic acid with the regression equation $C = 2.11 \times 10^{-2}A - 1.37$, where C is the cinnamic acid concentration expressed as µg l^{-1} h^{-1}, and A is the absorbance.

Gene expression analysis by real time—polymerase chain reaction

The transcription of genes involved in SA and JA mediated pathogen resistance (*PR1*, *ICS1*, *PDF1.2* and *AOS*) were studied with Real Time-PCR.

Table 1 Gene specific primers used to study *Ascophyllum nodosum* induced *Arabidopsis thaliana* resistance against *Pseudomonas syringae* pv. *tomato* DC3000

Gene	Gene locus	Gene specific primers
PR1	AT2G14610.1	F 5′ACATGTGGGTTAGCGAGAAG-3′
		R 5′ACTTTGGCACATCCGAGTCT-3′
ICS1	AT1G74710.1	F 5′ TTCTTCCGTGACCTTGATCC-3′
		R 5′ CCAAAAGGTTCCCATTCAAC-3′
PDF1.2	AT5G44420.1	F 5′-TGCTGGGAAGACATAGTTGC-3′
		R 5′-TGGTGGAAGCACAGAAGTTG-3′
AOS	AT5G42650.1	F 5′-TCATATCGCCGGAAAATCTC-3′
		R 5′-TTGAGGCATGTGTTGTGGTC-3′

(Table 1). Three-week-old plants were root treated with ANE C-ANE, E-ANE (1 g/l) or water control at the rate of 25 ml per plant. Plants were maintained for 48 h in a growth chamber at 22± 2°C and 16 day/8 night conditions and thereafter inoculated with *Pst* DC3000 ($0.01OD_{600}$). Plants were sampled at 6, 24 and 48 h after inoculation and total RNA was extracted with Trizol using the method of Chomczynski and Mackey (1995). Two μg of DNase treated RNA was reverse transcribed using a Retroscript kit (Ambion Inc., TX, USA) and the cDNA was purified using QIAquick PCR purification kit (Qiagen, Germany). Quantitative Real-Time PCR was performed with gene specific primers (Table 1) on StepOne™ Real-Time PCR System (Applied Biosystems) using SYBR green reagent (Roche Diagnostics, Mississauga, ON, Canada) following the manufacturer's instructions. Primer specificity was confirmed by observation of a single PCR product on 2% agarose gel and by melting curve analysis. 18S was used as an internal quantification control. Data were analyzed from two independent runs.

Data analysis

Data were analyzed using JMPIN statistical analysis software (SAS Institute, Inc.). Means at 95% confidence level were separated by Duncan's multiple range tests or Tukey's HSD test. Log transformation was applied when necessary to meet the criteria for analysis of variance. Microsoft Excel (Microsoft® Office Excel 2003, Microsoft, USA) was used for linear regression analysis for PAL estimation. Gene expression data were analyzed using relative expression values with StepOne™ Real-Time analysis software (Applied Biosystems).

Results

Ascophyllum nodosum extracts induced resistance against *Pst* DC3000 in Arabidopsis

We tested the *in planta* effect of the extracts on *Pst* DC3000 infection in Arabidopsis. Root treatment of Col-0 plants with *A. nodosum* extracts [aqueous (ANE), chloroform (C-ANE), and ethyl acetate (E-ANA)] reduced disease severity. On day four post-inoculation the disease severity was 57% in the control (water), 43% in ANE, 36% in C-ANE and 35% in E-ANE (Fig. 1a). The typical chlorosis symptoms of *Pst* DC3000 infection appeared 2 days after inoculation on control plants whereas the plants treated with *A. nodosum* extracts (ANE, C-ANE, E-ANE) showed delayed symptom development, restricting the infection to minor chlorosis at the site of inoculation (data not shown).

Further *Pst* DC3000 growth was suppressed in ANE treated plants as observed from the bacterial colony forming units (cfu) in the leaf tissues. *Pst* DC3000 colony forming units (cfu) were higher in the leaves of water control at all time points as compared to a significantly lower number of cfu's ($p<0.0001$) in *A. nodosum* extracts treatments (Fig. 1b).

Ascophyllum nodosum extract did not inhibit *Pst* DC3000 growth in vitro

To rule out direct anti-microbial activity of *A. nodosum* extracts, we investigated in vitro effect of *A. nodosum*

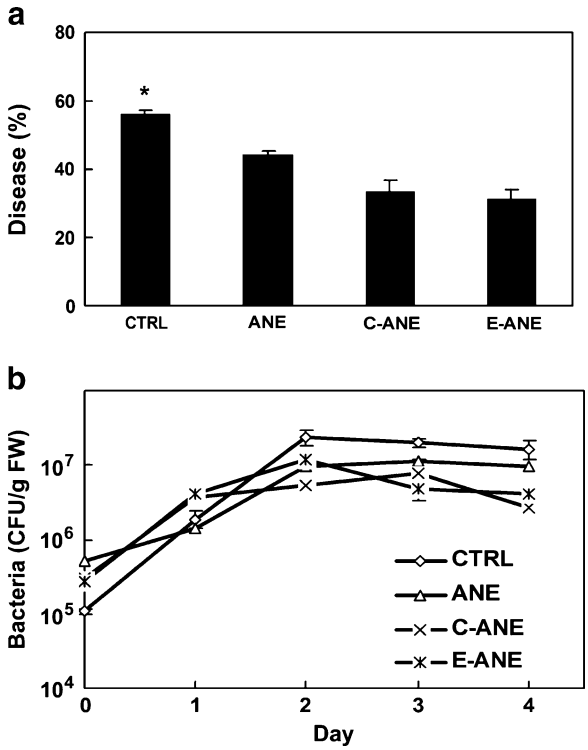

Fig. 1 *Ascophyllum nodosum* extracts induced resistance in *Arabidopsis thaliana* (Col-0) to *Pseudomonas syringae* pv *tomato* DC3000. Wild type Col-0 pretreated with *A. nodosum* extracts or water control (CTRL—water control; ANE—*A. nodosum* aqueous extract; C-ANE—Chloroform sub-fraction; E-ANE—ethyl acetate sub-fraction) were infiltrated with *Pst* DC3000 at 1×10^7 cfu/ml using a blunt syringe. Bacterial populations were determined from five leaves at 0, 1, 2, 3 and 4 days postinoculation. Results are the mean and standard error of bacterial populations (cfu/g FW). (**a**) Percent disease on plants treated with *A. nodosum* extracts compared to water control (**b**) Bacterial proliferation in *A. nodosum* extract treated plants as evidenced by colony forming units (cfu). Means marked with the asterisk are statistically different at the 5% confidence level based on Duncan's multiple range test. The experiment was repeated two times with similar results

extracts on *Pst* DC3000 growth. Extracts of *A. nodosum* did not exhibit inhibitory activity on growth of *Pst* DC3000 (Supplementary Fig. 1). In fact, bacterial growth increased by two-fold after 24 h of incubation in the broth containing aqueous extract of *A. nodosum* (ANE) probably due to high nutrient contents of seaweed extract. Both methanol and chloroform fractions showed marginal increases while the effect of the ethyl acetate fraction was similar to that of the control. These results are in contrast to in vivo effects of *A. nodosum* extracts on Arabidopsis resistance to the pathogen.

A. nodosum extract altered reaction of Arabidopsis mutants to *Pst* DC3000

We used Arabidopsis mutants to determine *A. nodosum* extracts-induced defence responses to *Pst* DC3000 infection. Water treated transgenic NahG plants were susceptible to bacterial infection and recorded a disease severity of 80% which was significantly higher ($P<0.05$) than disease severity on aqueous (ANE) or organic sub-fractions (C-ANE and E-ANE) (DI 30–33%) treated NahG plants (Fig. 2a). Similarly, the

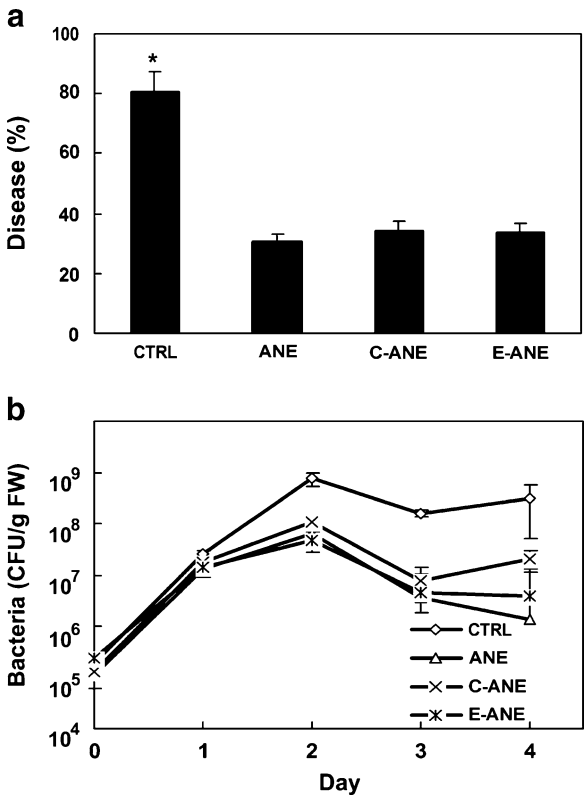

Fig. 2 Effect of *Ascophyllum nodosum* extracts on *Arabidopsis thaliana* mutant NahG response to *Pseudomonas syringae* pv *tomato* DC3000. NahG plants treated with *A. nodosum* extracts or water control (CTRL—water control; ANE—*A. nodosum* aqueous extract; C-ANE—Chloroform sub-fraction; E-ANE—ethyl acetate sub-fraction) were infiltrated with *Pst* DC3000 at 1×10^7 cfu/ml using a blunt syringe. Bacterial populations were determined from five leaves at 0, 1, 2, 3 and 4 days post-inoculation. Results are the mean and standard error of bacterial populations (cfu/g FW). (**a**) Percent disease on *A. nodosum* extracts treated NahG compared to control plants. (**b**) cfu in NahG plants treated with *A. nodosum* compared to control plants. Means marked with asterisk are statistically different at the 5% confidence level based on Duncan's multiple range test. The experiment was repeated twice with similar results

growth of *Pst* DC3000 (cfu) was more in water treated NahG plants compared to ANE,C-ANE and E-ANE treatments (Fig. 2b). This effect was significantly different ($P<0.05$) at 2 days after inoculation. Interestingly, *ics1*, a mutant with a defect in SA biosynthesis also exhibited moderate resistance to *Pst* DC3000 infection and the cfu at two, three and four d after inoculation were significantly lower than aqueous extract (ANE) treatment (Supplementary Fig. 2). In contrast, *A. nodosum* extracts did not affect the response of *jar1*, a mutant compromised in the JA dependent pathogen resistance, against *Pst* DC3000 infection (Fig. 3). The disease severity on *jar1* plants, root treated with the extracts of *A. nodosum,* ranged from 70% to 80% and it was not significantly different ($P>0.05$) from water treated plants (Fig. 3a). The bacterial growth in water treated plants was not different ($P>0.05$) from ANE treated plants at any time point after inoculation (Fig. 3b).

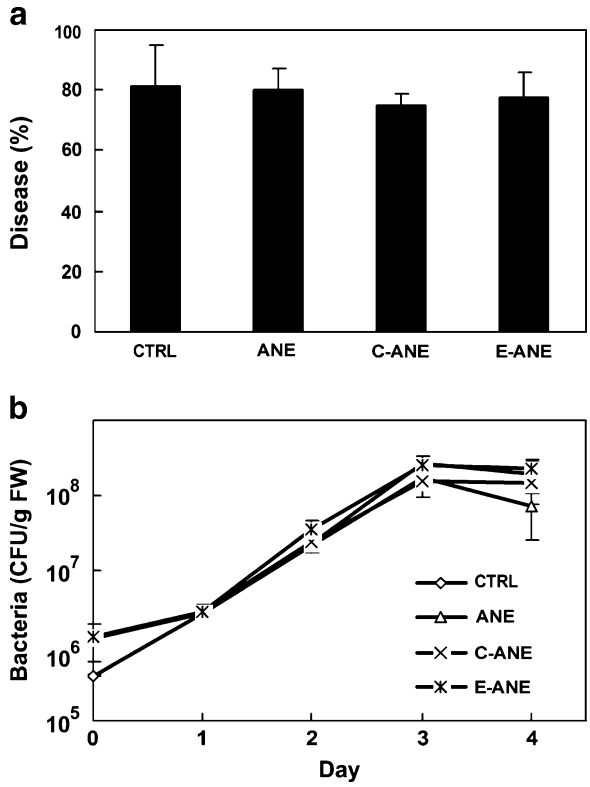

Fig. 3 Effect of *Ascophyllum nodosum* extracts on *Arabidopsis thaliana* mutant *jar1* response to *Pseudomonas syringae* pv *tomato* DC3000. The *jar1* plants treated with *A. nodosum* extracts or water control (CTRL—water control; ANE—*A. nodosum* aqueous extract; C-ANE—Chloroform sub-fraction; E-ANE—ethyl acetate sub-fraction) were infiltrated with *Pst* DC3000 at 1×10^7 cfu/ml using a blunt syringe. Bacterial populations were determined from five leaves at 0, 1, 2, 3 and 4 days postinoculation. Results are the mean and standard error of bacterial populations (cfu/g FW). **a** Percent disease on *jar1* on *A. nodosum* extracts treated plants as compared to control (water) plants. **b** The cfu in the leaves from *A. nodosum* extract or water (CTRL) treated *jar1* plants. Means marked with an asterisk are statistically different at the 5% confidence level based on Duncan's multiple range test. The experiment was repeated twice with similar results

Ascophyllum nodosum extract induced Arabidopsis resistance to *Sclerotinia sclerotiorum*

To determine if *Ascophyllum nodosum* extract induced Arabidopsis resistance to necrotrophic pathogens, *A. thaliana* Col-0 plants pre-treated through root and spray with aqueous extracts of *A. nodosum* (ANE) (1 g/l) were inoculated with *Sclerotinia sclerotiorum*. Pre-treatment with ANE enhanced the Arabidopsis resistance against *S. sclerotiorum* infection. There was delayed lesion development on the plants with a characteristic yellow halo developed around the lesions within 48 h after inoculation, with necrosis in the infected area (Fig. 4a, inset). The average lesion size on ANE treated plants at 48 h and 72 h after inoculation was significantly less ($P<0.05$) compared to control plants. The maximum lesion size on ANE treated plants was <4 mm whereas it was 6.6 mm at 72 h after inoculation on water treated plants.

We further tested the effect of aqueous *A. nodosum* (ANE) extract on the *S. sclerotiorum* pathogenicity factor, oxalic acid (OA) (Cessna et al. 2000) to determine if ANE treatment protects leaves against OA-induced injury. Fully open leaves of Col-0 plants were spotted with 10 and 25 mM of OA 48 h after ANE treatment and incubated overnight. The size of the OA induced lesions was significantly reduced with ANE treatment compared with water treated plants (Fig. 4b). The lesion size was significantly reduced ($P<0.05$) in leaves of ANE treated plants suggesting that OA induced toxicity was suppressed.

Histochemical analysis of PR::GUS and pAOS::GUS plants

To investigate the molecular defence response in Arabidopsis induced by *A. nodosum* extracts, the expression and localization of pathogenesis related protein 1 (*PR1*) and allene oxide synthase (*AOS*) gene

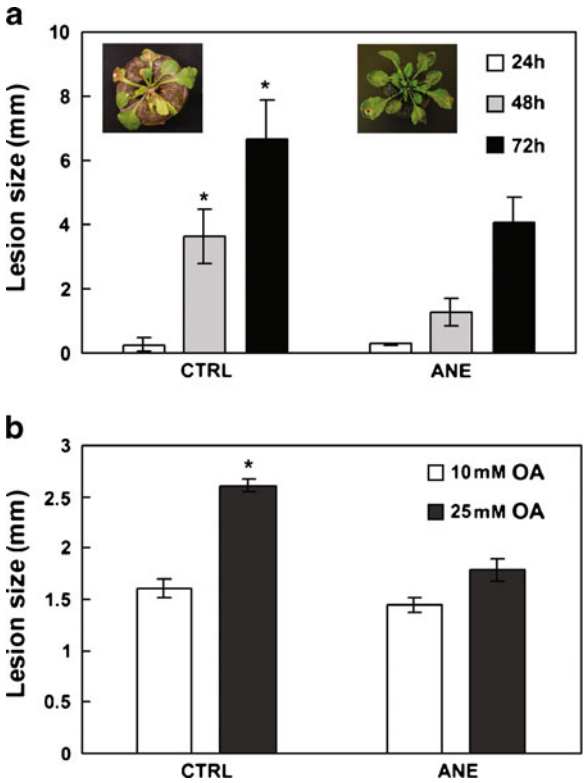

Fig. 4 Lesion size of *Arabidopsis thaliana* (Col-0) inoculated with *Sclerotinia sclerotiorum* after treatment with seaweed extracts (ANE). Col-0 plants treated with aqueous *A. nodosum* extracts or water control (CTRL—water control; ANE—*A. nodosum* aqueous extract) were inoculated with *S. sclerotiorum*. Lesion size (mm) was measured at 24, 48 and 72 h postinoculation. **a** Phenotype of Col-0 plants 72 h after inoculation with *S. sclerotiorum* (inset). Lesion size on *S. sclerotiorum* infected leaves collected from water (CTRL) or seaweed extract (ANE) treated plants at 24, 48 and 72 h after inoculation. Results are the mean of lesions from 15 plants; bars, ± standard error mean (SEM). **b** In vivo determination of the *S. Sclerotiorum* pathogenicity factor, oxalic acid (OA) on *A. thaliana* treated with water (CTRL) or ANE. Plants pretreated with ANE (1 g/l) were spotted with a 2 µl of oxalic acid (OA) with two (10 and 25 mM) different concentrations. Results are the mean of lesions from 10 plants; bars, ± standard error mean (SEM). Means marked with an asterisk are statistically different ($P < 0.05$) with Tukey's HSD test. The experiment was repeated twice with similar results

were determined using histochemical analysis of PR1::GUS (Uknes et al. 1992) and AOS::GUS plants (Kubigsteltig and Weiler 2003). Root treatment with INA (2, 6-dichloro-isonicotinic acid), an inducer of systemic acquired resistance, caused increased GUS activity in PR1::GUS plants at 48 h after treatment suggesting upregulation of *PR1* gene. The GUS was less induced in PR1::GUS plants treated with *A. nodosum* extracts suggesting that the *PR1* gene was not induced with seaweed extract treatment (Fig. 5). In contrast, the GUS activity was increased at 48 h in AOS::GUS plants treated with *A. nodosum* extracts suggesting that the JA biosynthesis gene *AOS* was induced (Fig. 5).

A. nodosum extract induced defence genes against *Pst* DC3000

To understand the defence pathways involved in *A. nodosum* extract induced resistance in Arabidopsis against *Pst*DC3000, we performed quantitative real time PCR of defense genes *PR1, ICS1 and PDF1-2* at 48 h after inoculation. The results revealed that *A. nodosum* extracts (ANE and C-ANE) elicited an increase in the transcript abundance of the JA dependent defence gene *PDF1.2* whereas the ethyl acetate extract did not (Fig. 6). The CHL extract also induced the transcript of *PDF1.2* in uninoculated plants. In contrast, control plants (water + 0.1% methanol) did not show a change in transcript *PDF1.2*. The expression of *AOS*, another gene that is important in JA dependent signalling, was found either at same or lower than basal level as observed in water control (data not shown). Interestingly, a strong induction of the transcript of *PR1* in ANE and a mild induction in E-ANE treated plants were observed at 48 h after inoculation. This induction was not observed with *Pst* DC3000 inoculation on control or CHL treated plants. *A. nodosum* extract did not affect the transcript abundance of the SA biosynthesis gene *ICS1,* and its expression was reduced with *A. nodosum* extract treatments compared to water control.

Discussion

In this paper, we showed that root treatment with *A. nodosum* extracts elicited defence responses in *A. thaliana* against a hemi-biotroph and a necrotrophic pathogen. The extracts of *A. nodosum* are widely used as biostimulants to promote growth and stress tolerance in plants. However, the mechanism(s) by which the *A. nodosum* extracts impart disease resistance are largely unknown. Current studies revealed that *A. nodosum* extracts when applied to Arabidopsis roots induced a jasmonic acid dependent

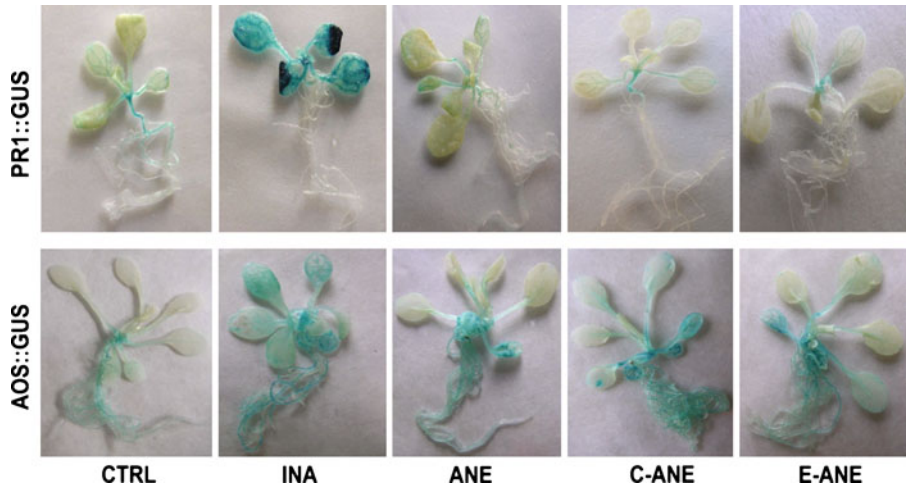

Fig. 5 The effect of *Ascophyllum nodosum* extracts treatment on the GUS expression in transgenic plants carrying PR1::GUS and AOS::GUS. Plants treated with the *A. nodosum* extracts for 48 were stained to visualize GUS activity and photographed with a digital camera. (CTRL—water control; INA—2, 6-dichloro-isonicotinic acid; ANE—*A. nodosum* aqueous extract; C-ANE—Chloroform sub-fraction; E-ANE—ethyl acetate sub-fraction). Results are representative of five plants and the experiment was repeated two times with similar results

systemic resistance in the leaves against *Pseudomonas syringae* pv. *tomato* DC3000.

NahG, a transgenic line of *Arabidopsis* that lacks the ability to accumulate SA and which is impaired in the expression of systemic acquired resistance, also exhibited a significant resistance when pre-treated with *A. nodosum* extracts. A similar trend was observed in *ics1* mutant suggesting *A. nodosum* extract induced resistance that was independent of salicylic acid. *A. nodosum* extract treatment did not alter the susceptibility of the *jar1* mutant. The *jar1* mutant which is impaired in rhizobacteria-induced systemic resistance, showed susceptibility to *Pst* DC3000 challenge (van Pieterse et al. 1998) mainly due to the inability to form JA-Ile conjugates that trigger the JA response (Staswick and Tiryaki 2004). These results, suggest that a JA dependent response is required in *A. nodosum* extract-induced disease resistance in Arabidopsis.

Pre-treatment of wild type *Arabidopsis* (Col-0) with aqueous extracts or organic sub-fractions of *A. nodosum* extract resulted in a significant reduction in disease severity and a reduced number of bacteria (cfu) in the leaf tissues. The differences in cfu in control from *A. nodosum* extract treated leaves were narrow; nevertheless the extracts were effective in suppressing the bacterial growth in leaves injected with a high level of inoculum (OD 0.01). Perhaps the leaf inoculation with low initial density of bacterium enhanced this difference as it affected disease symptom development (Katagiri et al. 2002; Meyer et al. 2005). Alternatively, the method of tissue infiltration could affect disease development (Meyer et al. 2005) in Arabidopsis.

Reduction in disease was primarily mediated by *A. nodosum* extract elicitation of physiological changes in the plant rather than via a direct anti-microbial effect on *Pst* as observed in in vitro assays. These results are quite surprising as many of the brown algae have been reported to have antibacterial activities (Schaeffer and Krylov 2000; Val et al. 2001) due to the presence of polyphenols (Zhang et al. 2006). This increase in growth was probably due to polysaccharides present in seaweed extracts that might have prebiotic effects or acted as a carbon source for the bacterium (Wu et al. 2007). Further, the concentration of antimicrobial compounds in the medium might be low, failing to suppress bacterial growth in the medium. In a similar study, *Ulva* extract was not antimicrobial against *Colletotrichum trifolii* development but it induced plant resistance against this pathogen (Cluzet, et al. 2004).

Induction of *PDF1.2* suggested a possible link to an increase in the *in planta* JA concentration. Among five plant defensin (*PDF*) genes reported in Arabidopsis, only *PDF*1.2 is induced upon pathogen challenge while others are constitutively expressed in various parts of the plant (Thomma et al. 2002).

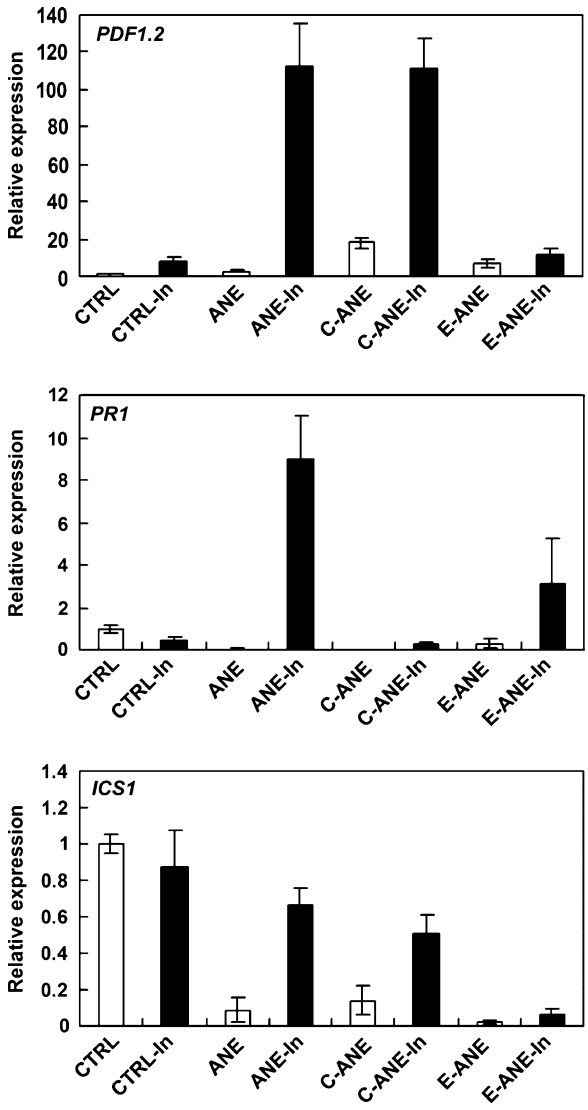

Fig. 6 Quantitative real-time reverse transcription polymerase chain reaction analysis of JA and SA dependent defence genes *PDF1.2*, *PR1* and *ICS1* in Arabidopsis plants treated with *Ascophyllum nodosum* extracts or water control (CTRL—water control; ANE—*A. nodosum* aqueous extract; C-ANE—Chloroform sub-fraction; E-ANE—ethyl acetate sub-fraction) at 48 h after *Pseudomonas syringae* pv *tomato* DC3000 inoculation. Total RNA was extracted from three plants with same treatment and were pooled for analysis and the experiment was repeated twice. 18S was used as internal quantification control. The fold change following infection is relative to transcript levels in a water-treated (CTRL) sample. Values shown are average of two independent runs. (In—Inoculated with *Pseudomonas syringae* pv *tomato* DC3000)

The activation of this marker gene by *A. nodosum* extracts support a JA dependent activation of systemic disease resistance. In addition, increased GUS expression in AOS::GUS plants suggests that JA dependent defence response was activated with *A. nodosum* extract treatment on Arabidopsis.

A. nodosum extract -induced defence in Arabidopsis against *Pst* DC3000 was largely SA independent . Although *PR1*, a pathogenesis related protein (Wildermuth et al. 2001), was mildly induced in treated plants, *ICS1*, the gene implicated in salicylic acid synthesis during systemic acquired resistance, did not change. This suggested that *A. nodosum* extract-induced resistance did not involve the SA pathway. In fact, *A. nodosum* extracts could have suppressed SA activity if the extracts contained the laminaran, a polysaccharide present in brown seaweeds, which suppressed SA accumulation (Mercier et al. 2001). Interestingly, the organic sub-fractions used in this study (C-ANE and E-ANE) do not contain laminaran suggesting that lipophilic components of *A. nodosum* extracts specifically elicited JA related defence responses in *A. thaliana*.

The seaweed extracts not only suppress the biotrophic pathogen *Pst*DC3000, but also protects against the necrotrophic pathogen, *Sclerotinia sclerotiorum*. Arabidopsis resistance to *S. sclerotiorum* is largely mediated by JA/ET, although a role for SA has also been proposed (Guo and Stotz 2007). In another study, the seaweed polysaccharide carrageenan was shown to enhance Arabidopsis resistance to *S. sclerotiorum* which was JA dependent (Sangha et al. 2010).

A closer look at the chemical constituents in these extracts revealed the presence of significant amount of fatty acids and sterols (Rayorath et al. 2008). *A nodosum* and other brown seaweeds are rich in fucosterol and fucosterol derivatives (Khan et al. 2009). Arabidopsis is known to contain a putative non-specific lipid transfer protein (nsLTP) that is involved in disease resistance mediated via JA and other oxylipin signals (Maldonado et al. 2002). nsLTPs bind to exogenous sterols thereby triggering disease signalling cascades (Cheng et al. 2004). JA, cholesterol and sitosterol are known to induce nsLTP mRNA in grapes infected by *Botrytis cinerea* (Gomès et al. 2003). It is plausible that sterols and fatty acids in the extracts could trigger nsLTPs in the plasma membrane that might potentiate disease resistance.

In summary, the results show that the extracts of the brown seaweed *A. nodosum* elicit systemic disease resistance in Arabidopsis against a hemi-biotroph and

a nectrotroph. Induction of systemic resistance has been proposed as an effective strategy to protect plants against pathogen attack. A number of chemicals such as INA (2, 6-dichloro-isonicotinic acid), BTH (benzothiadiazole) and BABA (β-aminobutyric acid) are known to elicit systemic acquired resistance in plants however, their cost of production is high (Edreva 2004). Unlike SAR, there is little information on bioactive compounds that elicit JA-mediated disease resistance in plants. The use of cost effective *A. nodosum* extracts offers alternate approaches of plant disease management. Additional research is needed to delineate the chemical nature of the active component(s) in *A. nodosum* that elicit JA dependent pathogen resistance.

Acknowledgements BP's lab is supported by funding from the Atlantic Canada Opportunities Agency, Acadian Seaplants Limited, Natural Sciences and Engineering Council of Canada and the Nova Scotia Department of Agriculture and Marketing. We thank Dr. Diane Cuppels, Agriculture and Agri-Food Canada, London, Ontario, Canada for providing the culture of *Pst* DC3000 used in this study, Dr. Xinian Dong for NahG seeds and Dr. Allan Shapiro for PR1::GUS transgene seeds and Dr. Innes Kubigsteltig for the seeds of pAOS::GUS transgenic line of *Arabidopsis*.

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