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Bioassay to detect *Ascophyllum nodosum* extract-induced cytokinin-like activity in *Arabidopsis thaliana*

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Abstract *Ascophyllum nodosum*, a brown macroalga, is the most widely used seaweed in agriculture. We report a rapid method for the detection of cytokinin-like activity in plants treated with a commercial *A. nodosum* liquid concentrate (Stimplex®) using a transgenic line of *Arabidopsis* carrying the ARR5 promoter fused to ß-glucuronidase (GUS) reporter gene. Based on GUS activity assay, an increase in cytokinin-like activity was detected in plants grown in vitro treated with 3 mL L^{-1} Stimplex®, whereas foliar spray treatments showed similar cytokinin-like activity at a concentration of 5 mL L^{-1}. Histochemical staining showed Stimplex®-induced GUS activity in leaf as well as in the root tissues. Taken together, our results suggest that Stimplex® contains compounds that may elicit endogenous cytokinin-like activity. Furthermore, it is shown that this bioassay can be used for rapid screening of extracts that can stimulate cytokinin-like activities using *Arabidopsis* AAR5::GUS reporter transgenic plants.

Keywords *Ascophyllum nodosum* · Cytokinin · Elicitor · *Arabidopsis thaliana* · Plant growth regulator

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Introduction

Brown algae (Phaeophyta) are among the most commonly used seaweeds in agriculture (Khan et al. 2009; Craigie 2010), and among them, *Ascophyllum nodosum* (L.) Le Jolis is the most researched seaweed (Taylor 1957; Baardseth 1970; Ugarte et al. 2006; Khan et al. 2009). Commonly known as rockweed, *A. nodosum* dominates the rocky intertidal shores of Atlantic Canada and Northern Europe (Taylor 1957; Ugarte et al. 2006, 2010). About 15 million metric tonnes of seaweed products are produced annually (FAO 2006), and a considerable portion of which is used in the production of plant nutrient supplements as well as biostimulants to enhance plant growth yield and productivity (Metha et al. 1967; Bokil et al. 1974; Crouch and Van Staden 1992; Craigie 2010). Positive effects of seaweed and seaweed products when applied to land plants and crops have been reported such as better nutrient uptake, enhanced plant growth, increased root development, and improved frost tolerance (Abetz 1980; Stirk and Van Staden 1997; Zodape 2001; Khan et al. 2009; Craigie 2010).

Arabidopsis response regulator 5 (ARR5) is a cytokinin primary response gene of *Arabidopsis*. ARR5 transcription rate, stimulated by cytokinin, has been studied using a fusion of the 59 regulatory sequences to the ß-glucuronidase (GUS) reporter gene (D'Agostino et al. 2000). ARR5 mRNA can be accumulated rapidly in response to cytokinin (Romanov et al. 2002). The aim of the present study was to use the ARR5::GUS reporter transgenic line of *Arabidopsis* for the detection of cytokinin-like activity in response to the application of Stimplex®, a commercial liquid extract of *A. nodosum*.

Materials and methods

Seeds of *Arabidopsis thaliana*, transgenic line CS25262, carrying 1.6 kb of the ARR5 gene promoter fused with

GUS reporter gene were obtained from *Arabidopsis* Biological Resource Centre, Ohio State University, Columbus, Ohio, USA. This transgenic line ARR5::GUS was used to detect cytokinin-like activity in response to Stimplex® in liquid culture as well as foliar spray treatments. The seeds were surface-sterilized in a 1.5-mL micro-centrifuge tube with 2% sodium hypochlorite with 5% Triton X-100 solution for 1 min and rinsed five times with sterile distilled water. Seeds were then left to dry on sterilized filter paper and then were evenly distributed on half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962; catalogue no. M5524; Sigma-Aldrich, USA), supplemented with 1% sucrose and 1% agar in Petri plates. The Petri plates were kept at 4°C in the dark for 2 days at 4°C to facilitate uniform germination, after which they were placed under a cool fluorescent light (100 μmol photons $m^{-2}s^{-1}$) with a 16:8-h photoperiod at 22±2°C for 7 days. After 5–7 days, the plants were approximately 2 cm long with well-differentiated roots and shoots; from these, uniform plants were selected and used for all the experiments.

Detection of cytokinin-like elicitor activity in liquid culture

"Stimplex®" liquid commercial concentrate of *A. nodosum* from Acadian Agritech, Acadian Seaplants Limited, Dartmouth, NS, Canada, was used. The working solutions of 1, 3, and 5 mL L^{-1} were prepared by dissolving the Stimplex® concentrate in sterile distilled water. The solution was then filter-sterilized using 0.22-μm SFCA syringe filters (Corning Inc., USA) and stored in sterile centrifuge tubes at 4°C until further use. *Arabidopsis* ARR5::GUS plants were grown on half-strength MS basal liquid medium for 7 days. Uniform seedlings were transferred to 12-well cell culture cluster plates (Corning, Cat. no. 3513) containing 1.5 mL of liquid half-strength MS basal medium supplemented with Stimplex® to a final concentration of 1, 3, and 5 mL L^{-1}, whereas 10^{-5} M 6-benzenylaminopurine (BAP) was used as a positive control. Plants treated with only half-strength MS basal liquid medium were used as the control. The plates were placed on an orbital shaker set at 90 rpm. Plants received light by placing them under a cool fluorescent light (100 μmol photons $m^{-2}s^{-1}$) with 16:8-h photoperiod at 22±2°C. Six seedlings from each treatment were used in a 12-well culture plate. After 48 h, the treated plants were removed and stained for GUS expression. The whole experiment was repeated three times.

Detection of cytokinin-like elicitor activity in foliar spray treatment

Arabidopsis ARR5-GUS seeds were grown on pre-soaked Jiffy-7 pellets (Jiffy Products, Canada). These were arranged on plastic trays and grown under greenhouse conditions as follows: temperature 22±2°C, 16:8-h photoperiod with 100 μmol photons $m^{-2}s^{-1}$ incident light, and 65% humidity. Following germination, the plants were irrigated every other day. After 3 weeks, the plants were treated with Stimplex® to a final concentration of 1, 3, and 5 mL L^{-1}, which were made up with distilled water containing 0.02% Tween 20. The young plants were sprayed with the solutions until dripping, with an atomizer. Each plant required about 1 mL of spray solution and 10^{-5} M BAP was used as a positive control. Plants sprayed with 0.02% Tween 20 served as the control. Individual, uniform-sized leaves of treated plants were removed after 48 h and stained for GUS expression.

Histochemical GUS staining

Histochemical GUS staining was performed by excising the leaves of foliar spray-treated plants after 48 h, whereas for liquid culture, the treated seedlings were incubated in 2-mL Eppendorf tubes containing GUS staining buffer [50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.1% (*v*/*v*) Triton X-100, 2% (*v*/*v*) dimethyl sulfoxide, and 2 mm 5-bromo-4-chloro-3-indolyl-D-glucuronide] kept at 37°C for 24 h (Jefferson and Wilson 1991). At the end of the incubation period, chlorophyll was removed by incubating in several changes of 100% ethanol, and the plants were photographed.

Quantification of GUS activity

To quantify the GUS activity in the leaves following Stimplex® treatment, a spectrophotometric assay method as described by Aich et al. (2001) was used. This method is based on the ability of GUS enzyme to hydrolyze *p*-nitrophenol ß-D-glucuronide (PNPG) to release yellow-colored *p*-nitrophenol (PNP). A standard concentration curve of PNP (Sigma-Aldrich) in 50 mM sodium phosphate buffer, pH 7.0, was prepared. Leaves from treated and control plants were excised after 48 h and used for the quantification of GUS activity. The enzyme extract was prepared from treated leaves by grinding leaf tissue (70–80 mg) in 200 μL of sodium phosphate buffer, pH 7.0, containing 10 mM mercaptoethanol, 1 mM EDTA, and 2% PVPP using a tissue macerator. The concentration of total protein in the enzyme extract was determined using the Bradford assay (Bradford 1976); the concentration was adjusted to 0.4 μg μL^{-1} of total protein before use. The GUS enzyme activity of treated *Arabidopsis* AAR5::GUS leaves after Stimplex® treatments were quantified using PNPG (a synthetic substrate) in six plants. The enzyme activity in Stimplex® treated leaves was quantified in units, where one unit is defined as 1 nM PNP $min^{-1}\mu g^{-1}$ protein.

To measure the GUS activity in the treated samples, 170 μL of reaction buffer (50 mM sodium phosphate buffer, pH 7.0, with 10 mM ß-mercaptoethanol), 20 μL of 10 mM PNPG substrate, and 10 μL of total protein extract were mixed in a 96-well plate and incubated at 37°C. The reaction was stopped by adding 0.4 M Na_2CO_3 (pH 11.0) and absorbance was measured at 405 nm. Each treatment consisted of six independent plants. Data were analyzed using Fisher's least significant difference test with $P \leq 0.05$ using CoStat statistical software (CoHort Software, Monterey, CA).

Results

In the present study, using a transgenic cytokinin response reporter line of *A. thaliana*, we were able to rapidly detect cytokinin-like activity enhanced by the application of Stimplex®, a commercial liquid extract of *A. nodosum*.

The liquid culture treatment of AAR::GUS plants with Stimplex® at a concentration of 3 mL L^{-1} resulted in increased GUS activity in the shoot as well as in root tissues (Fig. 1). The treatment of plants with a higher concentration of Stimplex® (5 mL L^{-1}) did not show increased GUS activity and were similar to the 3-mL L^{-1} treatment (data not shown).

The foliar spray of Stimplex® treatment showed that the maximum GUS expression was observed in 5-mL L^{-1} treated plants (Fig. 2), while lower concentrations (1 and 3 mL L^{-1}) resulted in lesser GUS expression. In contrast to treatment in liquid culture, we observed that Stimplex® in foliar treatment stimulated a similar response at 5 mL^{-1} as what we had observed at lower concentration (3 mL^{-1}) in liquid culture treatment. This is probably because in liquid culture, the seedlings were in constant contact with Stimplex® throughout the 48-h treatment period, which might have increased uptake of the compounds from the extract through roots and resulted in the induction of cytokinin-like activity in root and shoot tissues.

The appearance of GUS activity in leaf and root tissues of liquid culture plants suggested an increase of cytokinin-like activity in these tissues (Fig. 1). As expected, histochemical GUS staining showed that Stimplex® foliar spray increased cytokinin-like activity (Fig. 2). We also noticed that the GUS activity varied with the treatment concentration of Stimplex®. The staining results were in agreement with the results of the colorimetric quantification assay (Fig. 3). In all the experiments, BAP treatment resulted in the highest GUS activity (Figs. 1 and 3b).

In the case of foliar spray treatment, Stimplex® 5 mL L^{-1} produced the greatest response (Fig. 2). GUS enzyme assay results from leaves sampled after Stimplex® liquid culture treatments resulted in an increased GUS enzyme activity over the control plants (Figs. 1 and 3a, b). GUS enzyme activity was 2.55 units in Stimplex® 3 mL L^{-1} liquid culture plants compared to 2.17 units in the control plants. The positive control BAP (10^{-5} M), as expected, showed the highest GUS activity (3.49 units). Stimplex® stimulated approximately 17.5% higher cytokinin-like activity in the 5-mL L^{-1} treated plants compared to the control plants.

Discussion

Ascophyllum nodosum extracts have been used as a plant biostimulant in their various forms to improve crop performance and yield. Apart from containing naturally occurring plant growth stimulatory compounds such as vitamins, oligosaccharides, and micronutrients, they also contain phytohormones including those showing cytokinin-like activity (Zhang and Ervin 2004; Khan et al. 2009; Craigie 2010). In the present study, a rapid assay method was developed to detect and quantify the presence of cytokinin-like activity in response to Stimplex® treatment using a transgenic cytokinin response reporter line of *A. thaliana*. As ARR5 mRNAs accumulate rapidly in response to endogenous cytokinin (Romanov et al. 2002), we used

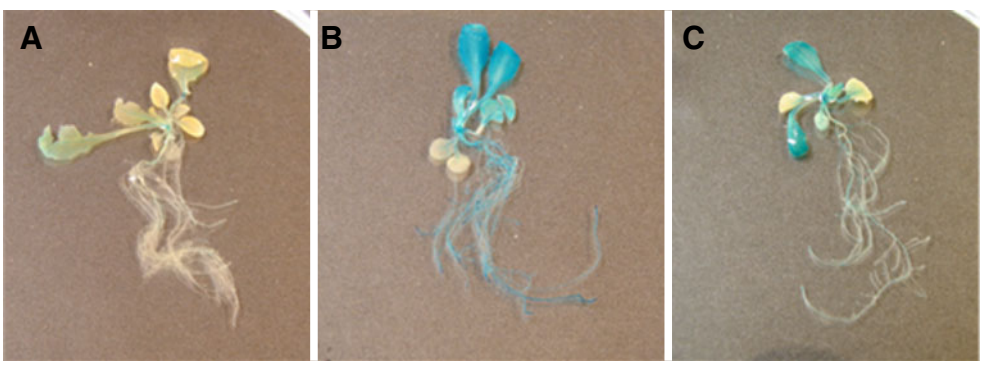

Fig. 1 Histochemical staining for β-glucuronidase (*GUS*) activity in ARR5::GUS transgenic *Arabidopsis* in liquid culture-treated seedlings: **a** water control, **b** positive control BAP 10^{-5} M, and **c** Stimplex® 3 mL L^{-1}

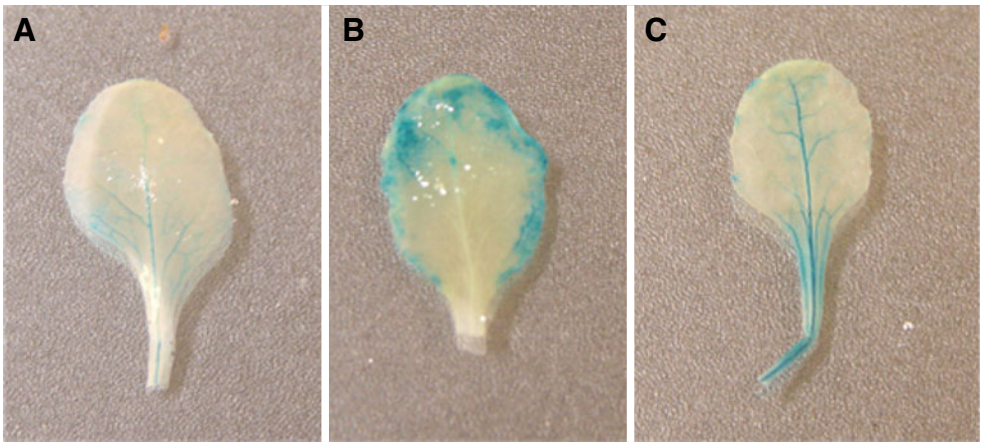

Fig. 2 Histochemical staining for β-glucuronidase (*GUS*) activity in ARR5::GUS transgenic *Arabidopsis* in foliar sprayed plants: **a** water control, **b** positive control BAP 10^{-5} M, and **c** Stimplex® 5 mL L^{-1}

the ARR5::GUS reporter line to detect if Stimplex® could stimulate cytokinin-like activity. Transgenic AAR::GUS plants when treated with Stimplex® in liquid culture at 3 mL L^{-1} concentration showed increased GUS activity in the shoot and root tissues. However, a higher concentration of 5 mL L^{-1} did not differ from the 3-mL L^{-1} treatment (data not shown). The foliar spray treatment also showed comparable increase in GUS expression, but at a higher concentration (5 mL L^{-1}). In comparison, the two methods of application resulted in a similar response; however, in the liquid culture, we observed that Stimplex® stimulated similar response at 3 mL L^{-1}, which was observed at a higher concentration (5 mL L^{-1}) in foliar treatment. The histochemical GUS staining assay in treated plant also confirmed that the treatment has resulted in increased cytokinin-like activity and was higher in the treated plants compared to the control plants. Furthermore, the GUS staining results were also in conformity with the colorimetric quantification results.

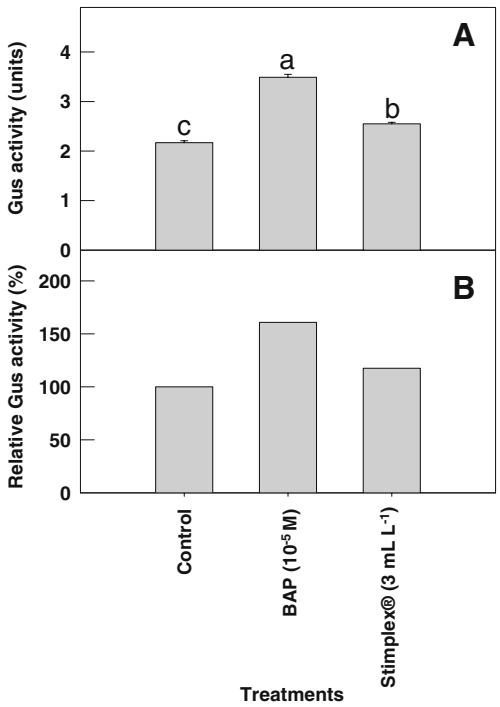

Fig. 3 **a** β-glucuronidase (*GUS*) activity in leaves of cytokinin-responsive *Arabidopsis* line after Stimplex® treatments in liquid culture. One unit is defined as enzyme activity required to release 1 nmolPNP min^{-1} μg^{-1} protein. **b** Percent increase in GUS activity in the leaves of cytokinin-responsive *Arabidopsis* line following Stimplex® treatment in liquid culture over control treatment. The values represent the mean of six plants (*bars* indicate standard error). The treatments were significantly different [$P \leq 0.05$; Fisher's least significant difference (*LSD*) test from the control]

The concentration of growth-stimulating compounds varies with the seaweed species, growth stage of the seaweed at harvest, and processing technology that is used to formulate the extracts (Khan et al. 2009; Craigie 2010). Using GC-MS, Sanderson and Jameson (1986) have previously shown cytokinin-like activity in a commercial extract of *A. nodosum* (Maxicrop) using tobacco callus bioassay. From their bioassay observations, they estimated about 5.4 μg kinetin equivalent activity per liter of the extract. Similarly, in Kelpak (*Ecklonia maxima*), Featonby-Smith and Van Staden (1983) estimated the total cytokinin activity equivalent of 516 ng kinetin in 20 g of Kelpak. However, Stirk and Van Staden (1997) stated that the use of bioassays to quantify the concentration of bioactive compound should be made with caution. Stirk et al. (2004) also reported that seaweed concentrates made from the kelp *E. maxima* and *Macrocystis pyrifera* also showed cytokinin-like activity. They found several different cytokinins in both the concentrates, and *trans*-zeatin-*O*-glucoside was the major cytokinin present in these extracts.

It is known that seaweed extracts (SWEs) in small quantities show positive effects on plant growth and development, suggesting that the active components present in SWEs are physiologically active at low concentrations (Stirk and Van Staden 1997; Khan et al. 2009; Craigie 2010). However, not all *A. nodosum* have a similar

composition because of the use of different starting raw materials as well as the different methods of processing (Rayorath et al. 2008). Nutrient components such as vitamins, antioxidants, amino acids, organic acids, complex minerals, macronutrients, and micronutrients have also been reported in seaweed extracts (Lobban and Harrison 1994; Khan et al. 2009; Craigie 2010). Nonetheless, nutrients in SWEs at such low concentrations cannot possibly cause pronounced effects that are associated with growth and development following SWE applications. For this reason, plant growth regulators (PGRs) such as cytokinin have been proposed to be associated with the growth-promoting activity of SWE application as exogenous application of PGRs elicits a similar response. However, it is important to note that more than one group of PGR have been implicated due to the wide range of physiological responses observed following the application of SWEs (Crouch and van Staden 1993; Khan et al. 2009). Cytokinins promote cell division and proliferation, boosting the sink activity of roots, resulting in growth stimulation (Nelson and Van Staden 1984a, b). Interestingly, the root growth-promoting activity of kelp extract (made from *E. maxima*) was observed when applied to the roots or as a foliar spray (Finnie and Van Staden 1985).

Our results showed that Stimplex® treatment resulted in increased cytokinin-like responses in *A. thaliana* plants, which suggests that the extracts have compounds that may have contributed to cytokinin-like activity. On the other hand, Stimplex® may contain compound(s) that may trigger endogenous synthesis of cytokinin-like compounds, leading to higher cytokinin concentration in plant tissues. The cytokinins that have been reported in seaweeds include *trans*-zeatin, *trans*-zeatin riboside, and their dihydro derivatives (Stirk and van Staden 1997). Stirk et al. (2003), using LC/MS analysis of 31 seaweed species, showed that cytokinin profiles were similar in all the macroalgae regardless of their taxonomy and growing locality and belonged to various groups, showing that Z and isopentenyladenine (iP) conjugates of cytokinins were the predominant cytokinins. They found that the major type of isoprenoid cytokinins was zeatins, with *cis* forms being the more prevalent than *trans* forms and iP derivatives. However, a small number of dihydrozeatin-type cytokinins were found at very low levels in only nine species. They also indicated that aromatic cytokinins such as benzyladenine (BA) and *ortho*- and *meta*-topolin derivatives were also present, but to a lesser extent; however, the topolins were higher in concentration and diversity than BA. The seaweed concentrates made from the kelps (*E. maxima* and *M. pyrifera*) have been shown to exhibit cytokinin-like activity. Several cytokinins were present in both concentrates, and among them, *trans*-zeatin-*O*-glucoside was the main cytokinin present in these extracts (Stirk et al. 2004).

The soybean callus bioassay has been used to detect and quantify cytokinin-like activity in commercial SWEs (Stirk and Van Staden 1997), where the commercial product Seamac (an *A. nodosum* extract product) showed maximum callus growth among the six commercial products used in the study. Although the soybean callus bioassay is widely used for the detection of cytokinin-like activity in SWE, this technique is time-consuming (4–5 weeks) and labor-intensive, which limits the number of samples that can be analyzed at a given point of time. The method reported in this study using the *A. thaliana* ARR5::GUS reporter is advantageous because the method is rapid, and it is less laborious to detect and quantify the cytokinin-like activity in response to SWEs.

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