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Increased freezing stress tolerance of *Nicotiana tabacum* L. cv. Bright Yellow-2 cell cultures with the medium addition of *Ascophyllum nodosum* (L.) Le Jolis extract

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Abstract

The study of bioactive components of the brown alga *Ascophyllum nodosum* (L.) Le Jolis has shown that they promote growth and increase productivity of plants. However, a standardized model system providing consistent responses to such bioactive components has yet to be established. Given that freezing stress, especially in northern climates, reduces plant growth and productivity, a technique was developed to protect plant cells under freezing stress using the natural products made from the abundant *A. nodosum* as a cell culture supplement. In this study, a homogenous cell culture system of *Nicotiana tabacum* L. cultivar Bright Yellow-2 (BY-2) suspension cells was used to investigate the bioactivity and protection level of this alga extract under freezing temperatures, and BY-2 cell growth, physiology, and molecular changes were measured in the presence or absence of the extract. The results indicated that *A. nodosum* extract significantly improved BY-2 cell survival after exposure to freezing temperatures. Inclusion of alga extract also improved cell growth, membrane stability, and nuclear integrity, and reduced cell death of cold-stressed BY-2 suspension cultures. It was concluded that *A. nodosum* extract influenced cellular and molecular regulation and triggered mechanisms, such as osmolyte accumulation and antioxidant activity, to combat freezing stress in BY-2 suspension cells.

Keywords Freezing stress · *Ascophyllum nodosum* (L.) Le Jolis extract · Suspension culture · Growth · *Nicotiana tabacum* L. cv. Bright Yellow-2

Introduction

Rapid changes in environmental conditions pose a serious problem for plant productivity. In crops, for example, environmental stress can decrease yield potential by 50% or more (Moghadam *et al.* 2013). Freezing is a critical environmental concern for plants, especially in northern climates (Miura and Furumoto 2013). Freezing stress affects plants differently, based on a variety of factors, such as stress condition and plant type (Mullet and Whisitt 1996). The fluids within plant intercellular spaces contain lower concentrations of solute than intracellular fluids, and thus have a higher freezing temperature. Crystallization of ice starts at sub-zero temperatures in these extracellular spaces, resulting in dehydration as a consequence of the transfer of cellular water to the extracellular spaces, which in turn can cause degradation of proteins and severe cell damage (Thomashow 1998; Hasanuzzaman *et al.* 2013).

The plasma membrane of plants serves as the site for receiving and transducing signals of environmental stress and is the primary site of freezing injury. The occurrence of phase transition in the unsaturated membrane lipids (such as phosphatidylcholine and phosphatidylglycerol) and the fluidity of plant cell membranes minimize the cell damage resulting from freezing (Jan and Andrabi 2009; Rayirath *et al.* 2009). However, membrane permeability can be affected as lipid molecules shift their phase from fluid to a gel-like (semi-solid) form. These changes may result in ion leakage from

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the membrane, eventual cell dysfunction, and subsequent plant death (Sinha *et al.* 2015).

The significance of stress tolerance in plant productivity led to several studies in recent years (Rayirath *et al.* 2009; Qin *et al.* 2011; Nair *et al.* 2012). Traditional plant breeding methods, for example, by selecting stress tolerant cultivars, and biotechnology-based techniques, such as gene cloning to develop freezing tolerant cultivars (Guo *et al.* 2006), have reduced the impact of environmental stress to some extent, by improving plant survival (Rayirath *et al.* 2009). Other approaches to improve plant productivity and reduce the effects of environmental stress on crop plants used in modern agriculture often depend on the use of fertilizers or chemical additives. However, in order to reduce certain harmful side effects from fertilizers and agricultural-chemicals, the use of natural products for increasing productivity and diminishing effects of stress is sought after and can be an environmentally friendly and economically practical (Battacharyya *et al.* 2015).

The positive effects of the brown alga *Ascophyllum nodosum* (L.) Le Jol. on germination, growth, shelf life, yield, and the quality of crops, *e.g.*, onion, potato, and lettuce, have been known to farmers for decades (Nabati 1991; Nabati *et al.* 1994; Battacharyya *et al.* 2015). The extracts made of *A. nodosum* contain a range of macro- and micro-nutrients and show their effects at very low concentrations (*e.g.*, 1 to 10 g L^{-1}) in the field (Ali *et al.* 2016; Santaniello *et al.* 2017; Melo *et al.* 2018). It has been shown that algal extracts can be used to lower the harmful effects of environmental stress on plants (Khan *et al.* 2009; Rayirath *et al.* 2009; Karunatilleke 2014). Hence, the use of algal extracts to combat freezing stress could be an attractive alternative for plant growers and researchers, which provided the impetus for the present study.

The establishment of a standardized model system for evaluating freezing stress was required so that the effects of various growth stages or of physiological diversity of a plant species on research observations could be minimized. To this end, a fast-growing plant cell line, *Nicotiana tabacum* L. (tobacco) cultivar Bright Yellow-2 (BY-2) suspension cells was selected to help develop a bioassay system for indexing the bioactivity of algal extract. The objective of the present study was to identify whether and how algal extract was effective in protecting BY-2 cells from the detrimental effects of freezing stress. The current study constituted the first attempt to examine the effect of algal extract on cold-stressed BY-2 cell survival.

Materials and Methods

Cell culture conditions The BY-2 cells were a kind gift from Dr. Samuel, University of British Columbia, Canada. Cells were cultured in LS (Linsmaier and Skoog 1965) growth medium (PhytoTechnology Laboratories®, Lenexa, KS). The growth medium was adjusted to pH 7 using 1 N NaOH and autoclaved at 120°C for 20 min. The medium was fortified with 370 mg L^{-1} KH_2PO_4, 0.2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg L^{-1} myoinositol, and 1 mg L^{-1} thiamine hydrochloride (Sigma-Aldrich®, St. Louis, MO). Thiamine hydrochloride was filter sterilized and added to the medium after autoclaving. A New Brunswick® shaker incubator Innova 42R (Eppendorf, Hauppuage, NY) was set at 130 rpm and kept at 27°C in the dark for cells to grow. The cells were grown in 125-mL Erlenmeyer flasks containing 30 mL of medium. Cell suspensions in the stationary growth phase were subcultured weekly with a 1:15 dilution (Nagata *et al.* 1992).

The powdered alkaline extract of *Ascophyllum nodosum* (L.) Le Jol is commercially available and was provided by Acadian Seaplants Limited, Darmouth, NS, Canada. An aqueous solution of the algal extract was prepared as a stock solution by dissolving 0.1 g of powdered extract in 10 mL of distilled water. The solution was filtered sterilized using a 0.2-μm syringe filter (Pall Corporation, Port Washington, NY) and stored at 4°C for further use (Rayirath *et al.* 2009). To treat the cells with algal extract, cell aliquots (100,000 to 120,000 cells mL^{-1}) were cultured in six-well plates at the final volume 4 mL culture per well (Corning Incorporated, Corning, NY). The BY-2 cells were then treated with *A. nodosum* extract in 0.01, 0.05, or 0.1 g L^{-1} concentrations. In control cultures, water was used and added into the culture. The six-well plates were kept in the shaker incubator set at 130 rpm, at 27°C in the dark. The BY-2 suspensions remained in the plates for 7 d and were freeze-stressed at conditions described below.

Freezing stress assessment Freezing conditions of 0°C, − 3°C, or − 5°C were applied to reveal how *A. nodosum* extract might mediate freeze tolerance of BY-2 cells. After 3 d of growth at 27°C, the cell plates were transferred to a low-temperature incubator for 24 h to apply the stress. After 24 h, the cultures were transferred back to the 27°C incubator for a 3 d recovery stage. The dry weight of cells was measured to determine the growth rate before, during, and after the cold stress. Each experiment for each condition (optimum temperature and cold stressed) was conducted in three biological and technical replicates. At stress condition, for example, each stage of cold stress (before, during, and after stress) contained nine wells as three replicates. To sample the cells, the whole culture volume was collected at each stage. However, there was a parallel culture growing at the same time for next stage. The large number of replicates minimized the technical error. A schematic diagram of the growth experiment is shown in Fig. 1. Growth assessment was measured as weight of cells. In brief, cell suspensions were filtered through a Whatman filter paper, 75 mm diameter, Grade 1: 11 μm (Sigma-Aldrich®) of known weight; the weight of filtered cells was recorded as fresh

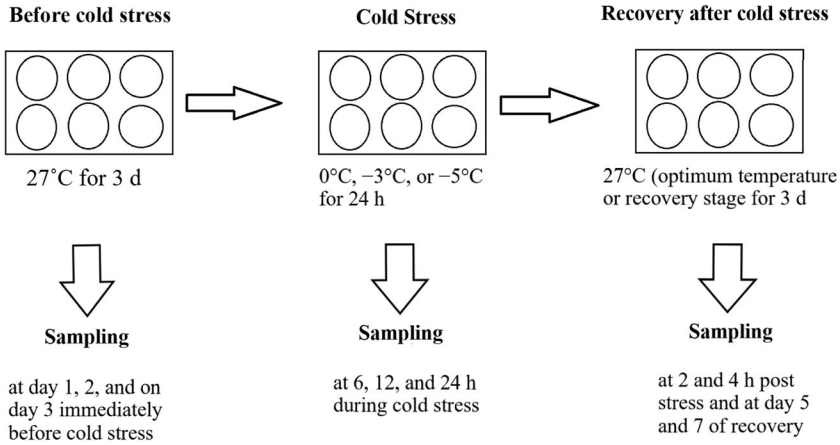

Figure 1. A schematic representation of the freezing stress experiments. *Nicotiana tabacum* cv BY-2 cells were sampled before the stress, on days 1, 2, and 3 (sampling on day 3 occurred just before the cold stress); during the stress, at 6 h, 12 h, and 24 h of cold; and at 2 h, 6 h, and at days 5 and 7 of recovery after freezing stress. Different assays such as cell growth, cell viability, ion leakage, and gene expression were conducted. For ion leakage and gene expression analyses, time points were selected to record cell responses at the earliest point possible.

weight. Thereafter, cells were dried overnight in an oven at 60°C, and then the filter papers containing dried cells were kept in desiccator before weighting to prevent absorbing moisture; the dry weight of the cells was determined by subtracting the weight of the filter paper from the weight of the dried cells with the filter paper and was expressed in milligrams per milliliter sampled.

Cell viability assay Cell viability was quantified *via* establishing cell numbers using a hemocytometer. Viable cells were observed and counted by 3,3-[3,3-dimethyl-4,4-biphenylylene) bis(azo)]bis(5-amino-4-hydroxyl-2,7-naphthalenedisulfonic acid) tetra sodium salt (Trypan blue) exclusion staining under a light microscope. In brief, Trypan blue was dissolved in water at a final concentration of 0.4 g L^{-1}. To stain the cells, 20 μL of Trypan blue solution was added into 500 μL of the BY-2 cells. The suspension was spun down at 1000 rpm for 2 min (Eppendorf, 5424R, Hamburg, Germany), then the supernatant was removed, and the remaining cells were de-stained with distilled water to remove the background stain and were observed under the microscope with × 10 objective lens. Cells that were not stained blue were counted as viable cells (De Pinto *et al.* 1999; de Pinto *et al.* 2002).

Membrane permeability test To test for possible membrane damage due to freezing, the lipophilic membrane dye 9-diethylamino-5H-benzo([α]alpha)phenoxazine-5-one (Nile red) was used (Greenspan *et al.* 1985). Nile red was first dissolved in acetone (1 mg mL^{-1}) before water was added to dilute the stain to the 0.01 μg mL^{-1} final concentration. For microscopic observation, the stained cells were suspended in pH 7 phosphate saline buffer. Afterwards, the cells were observed with an Olympus DP80, BX63F fluorescent microscope (excitation 450 to 500 nm and emission 529 nm) (Olympus Corp., Tokyo, Japan).

Cell nucleus damage under freezing The morphology of the BY-2 nuclei under freezing stress was observed. For this purpose, nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI). Cell suspensions were fixed in 100 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) at pH 6.8, 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA), 10 mM $MgSO_4$; with 4% (*v*/*v*) formaldehyde as fixative (PEM buffer), for 30 min (De Pinto *et al.* 2002) (Sigma-Aldrich®). Then, PEM buffer with 1 μg mL^{-1} of DAPI and 0.2% (*v*/*v*) Triton™ X-100 was used to stain the cells, which were observed with an Olympus DP80, BX63F fluorescence microscope (excitation 359 nm and emission 461 nm) (Olympus Corp.).

Cell membrane leakage test Ion leakage of the membrane was measured according to Liu *et al.* (2010). A LCO 302 conductivity meter (VWR, Radnor, PA) was used to measure the BY-2 suspension cell conductivity, and was recorded as C1. The same cell suspension was then boiled for 30 min and the conductivity was measured as C2. The relative ion leakage was determined as (C1 ÷ C2) × 100 (Zhao *et al.* 2008).

Quantitative real-time polymerase chain reaction The differential expression of *fucosyltransferase*, *betaine aldehyde dehydrogenase 1*, *galactinol synthase 2*, *glutathione S-transferase*, *activating protein 2*, *pyrroline-5-carboxylate synthase*, *acetyl-CoA carboxylase*, and *digalactosyldiacylglycerol* genes involved in low temperature stress response was analyzed to discern the fundamental role of *A. nodosum* extract in freezing stress tolerance. The primers used for the quantitative real-time polymerase chain reaction (qRT-PCR) experiment are shown in Table S1. Cells supplemented with 0.1 mg mL^{-1} of *A. nodosum* extract from the freezing stress experiment at − 5°C were sampled and were used for qRT-PCR. Cell samples were taken at day 3, without

the stress, 12 h after starting the stress, day 4 during the stress, and 2 and 6 h after withdrawing the stress (during recovery stage) (see Fig. 1).

Extraction of RNA was performed using TRIzol® reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instruction (Kit Cat No: 15596026; Invitrogen). The harvested cells (4 mL) were centrifuged for 2 min at 4°C, the media were removed, and the TRIzol® reagent was added to lysis the cells by pipetting the solution several times. One microgram of the extracted RNA was used for DNase treatment using Ambion® DNA-free™ kit (Thermo Fisher Scientific®, Waltham, MA), and first-strand cDNA was synthesized using the Reverse Transcription Kit (Applied Biosystem, Foster, CA). qRT-PCR was carried out in a "Step One *Plus*® Real-Time PCR System" (Applied Biosystems) using GoTaq® qPCR Master Mix (Promega, Madison, WI) adopting the manufacturer's instructions in 50-μL reaction volume. The data were normalized with *elongation factor-1α* as the internal standard. The results were used to evaluate the fold change in transcripts of selected genes in cells with algal treatment as well as controls (water treated cells) under freezing stress. The experiment was conducted in triplicate. The analysis of data was done by Step One software version 2.1 (Applied Biosystems) with the ΔΔCT algorithm used to calculate the relative amount of mRNA.

Statistical analysis The experiment was conducted in triplicate. Three plates (containing 9 wells in total), each well contained 4-mL cell culture constituted as the technical replicates; the entire experiment was repeated three times; the number of replicates was the same under all conditions such as optimum temperature, freezing stress (0°C, −3°C, or −5°C), extract treatments, and controls (water-treated cells) throughout the experiments. The *A. nodosum* extract was used in three different concentrations (0.01, 0.05, or 0.1 g L^{-1}) for all assays except qRT-PCR test in which only one concentration of *A. nodosum* extract (0.1 g L^{-1}) was used. The control cultures were grown under identical condition as to algal-treated cells except that they received the same volume of water instead of *A. nodosum* extract. The data were analyzed as variance comparison and compared to the controls. Analysis of variance (ANOVA) was carried out using Tukey test (honestly significant difference, HSD) at $P \leq 0.05$ with Minitab® software, version 17.3 (Minitab Inc., State College, PA).

Results and Discussion

***A. nodosum* extract affected growth of BY-2 cells in optimum temperature condition** Comparison of means showed that the cultures treated with 0.01 or 0.05 g L^{-1} *A. nodosum* extract were not significantly different from the control culture in terms of growth (Fig. 2). However, increasing the concentration of *A. nodosum* extract to 0.1 g L^{-1} significantly decreased BY-2 growth, as indicated by reduced dry weight (Fig. 2). It was hypothesized that the adverse effect of *A. nodosum* extract on BY-2 growth would be eliminated once culture temperature decreased to stressful levels, as previous studies have indicated that *A. nodosum* extract improves plant growth and productivity under stressful conditions (Rayirath *et al.* 2009; Karunatilleke 2014). Therefore, freezing stress was applied to BY-2 suspension cultures to further study of *A. nodosum* extract effect on the cell growth.

The cells treated with *A. nodosum* extract showed a higher cold stress tolerance Under freezing stress, the *A. nodosum* extract-treated cells achieved higher dry weights than control cells (Fig. 3*a–c*). Interestingly, the effect of *A. nodosum* extract on cell protection and/or growth was highly dependent

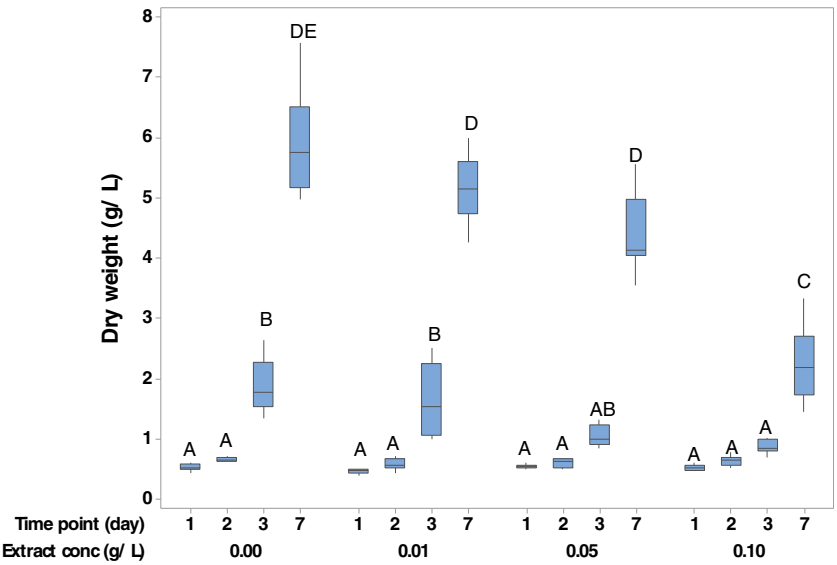

Figure 2. Effects of 0, 0.01, 0.05, or 0.1 g L^{-1} of *Ascophyllum nodosum* extract on growth (measured in dry weight) of *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) BY-2 suspension cells grown at 27°C. Dry weight *means* (± SD) with *different letters* represent significant differences among the concentrations of *A. nodosum* extract at $\alpha <$ 0.05 by Tukey test. *Asco conc*, *A. nodosum* extract concentration.

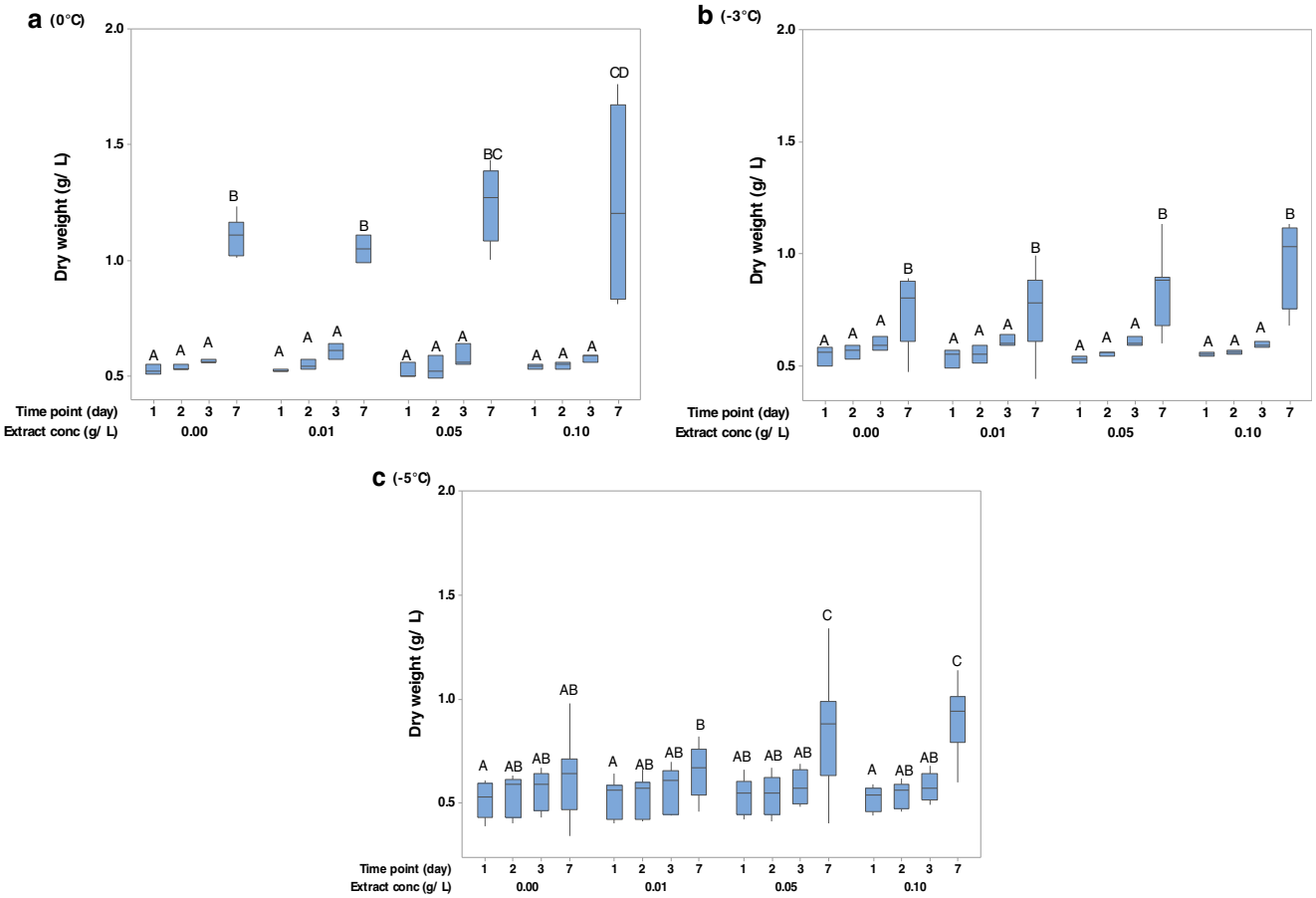

Figure 3. Effects of 0, 0.01, 0.05, or 0.1 g L^{-1} of *Ascophyllum nodosum* extract on growth (measured in dry weight) of *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) suspension cells under freezing temperatures (*a*) 0°C, (*b*) − 3°C, and (*c*) − 5°C. Dry weight *means* (± SD) with *different letters* represent significant differences between the concentrations of *A. nodosum* extract at $\alpha < 0.05$ by Tukey test. *Asco conc*, *A. nodosum* extract concentration.

on the concentration of the extract used in the experiment. For example, at a temperature of − 5°C (Fig. 3*c*), the dry weight was the highest when cells were treated with 0.1 g L^{-1} *A. nodosum* extract, compared to that with 0.01 and 0.05 g L^{-1} of the extract. Thus, unlike the effect at 27°C, *A. nodosum* extract enhanced the growth of cells under freezing stress. A similar scenario was observed in the cultures' growth at temperatures 0°C and − 3°C (Fig. 3*a*, *b*, respectively). However, at the latter temperatures, statistical analysis showed that the growth of the extract-treated cells was not significantly higher than that of the controls (Fig. 3*a*, *b*), except for the 0.1 g L^{-1} extract-treated group at 0°C (Fig. 3*a*). At this point, the question was posed, whether *A. nodosum* extract induced cell growth or prevented unfavorable effects of the stress. To discriminate between these possibilities, a cell viability test was conducted.

***A. nodosum* extract reduced cell damage resulting from freezing stress** Cell death accompanied by plasma membrane shrinkage has been reported as a sign of programmed cell death (PCD; Reape and McCabe 2013). A viability test using Trypan blue exclusion was performed to determine whether *A. nodosum* extract induced cell growth improvement by decreasing the number of dead cells under freezing stress. At 0°C, cell viability decreased to 54 and 52% immediately after cold treatment (3.5 d) in BY-2 suspensions treated with the 0.05 and 0.1 g L^{-1} of *A. nodosum* extract, respectively, and rebounded slightly during recovery, to about 77 and 67% cell viability, respectively (Fig. 4*a*). Furthermore, upon recovery from this temperature at 7 d, 53% viability was observed in cultures supplemented with 0.01 g L^{-1} of *A. nodosum* extract, as well as in the controls (Fig. 4*a*). Cultures exposed to − 3°C (Fig. 4*b*) and supplemented with 0.1 g L^{-1} of the extract showed almost 81% cell viability at 7 d. However, at this temperature, the viability of cells was closer to 23% in both, the control and the 0.01 g L^{-1} treatment (Fig. 4*b*). Surprisingly, at temperature − 5°C, cell viability was around 77% for the cultures treated with 0.1 g L^{-1} of *A. nodosum* extract (Fig. 4*c*). Conversely, cell viability was less than 20% in the control and the cultures with 0.01 g L^{-1} of *A. nodosum* extract for the − 5°C cold-treated cells (Fig. 4*c*).

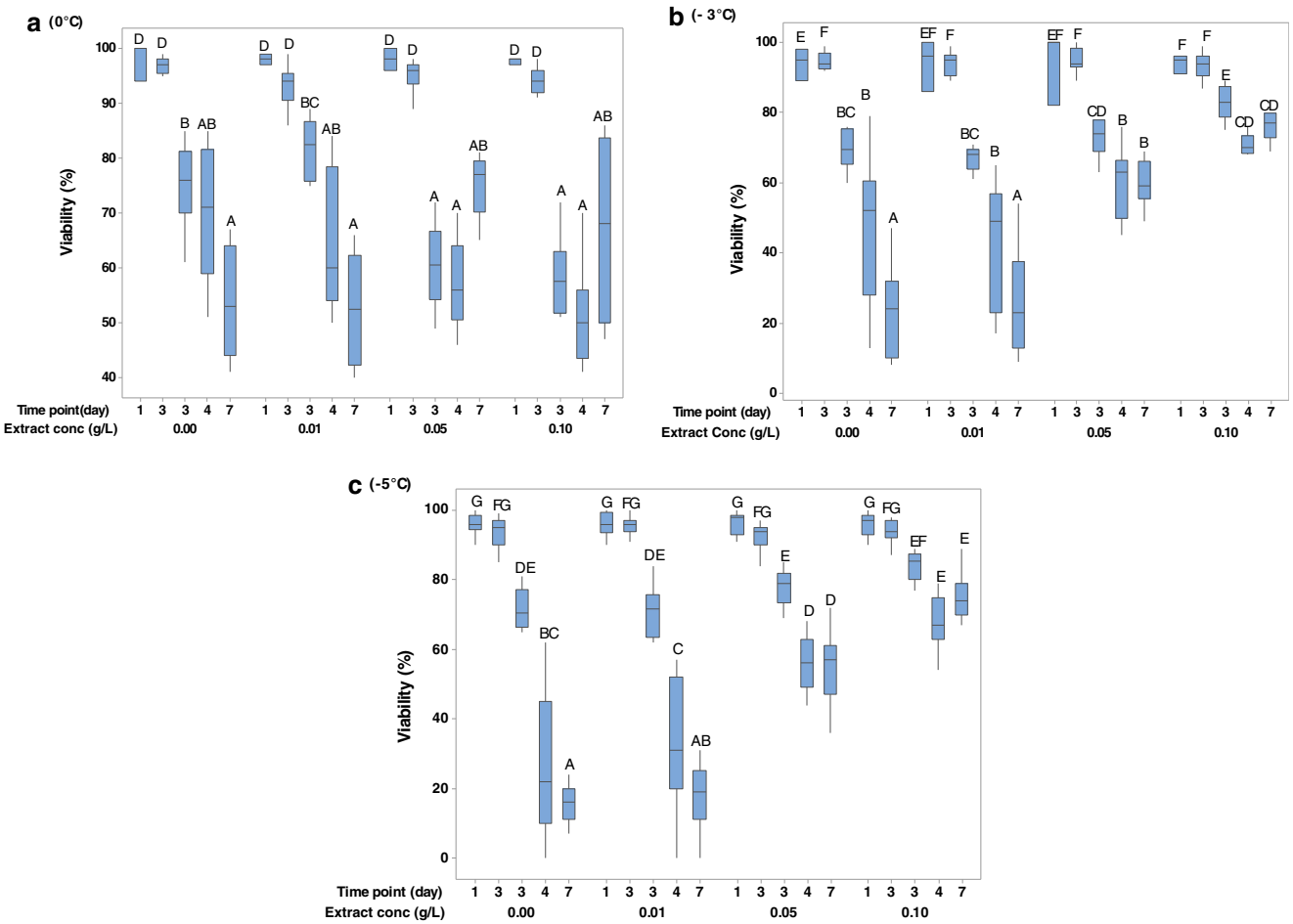

Figure 4. Viability assessment by Trypan blue exclusion staining of *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) cells treated with 0, 0.01, 0.05, or 0.1 g L^{-1} of *Ascophyllum nodosum* extract under freezing stress of (*a*) 0°C, (*b*) − 3°C, and (*c*) − 5°C. *Means* (± SD) with *different letters* represent significant differences between the concentrations of *A. nodosum* extract at $\alpha < 0.05$ by Tukey test. *Asco conc*, *A. nodosum* extract concentration.

The viability experiment verified that *A. nodosum* extract treatment enhanced the stress tolerance of the BY-2 cells, resulting in protection of cells from stress damage, whereas, freezing stress led to irreversible cell damage in control condition. Cultures treated with 0.1 g L^{-1} of *A. nodosum* extract showed a considerable increase in viability. For each of the three temperatures studied, day 4 was critical as a tremendous amount of cell deaths was observed. The reason for this huge viability loss accompanying the rapid change of temperature could be attributed to a sudden change in osmotic pressure that led to cell death (Wolfe and Bryant 1999; Rayirath *et al.* 2009; Fig. 4). Figure 5*a–d* show BY-2 suspension cultures grown in medium treated with 0, 0.01, 0.05, and 0.1 g L^{-1} concentrations respectively of *A. nodosum* extract at − 5°C after Trypan blue exclusion staining, with dark blue cells denoting nonviable, dead cells.

A. nodosum extract protected the stressed BY-2 cells

Temperatures at or below freezing may cause oxidative stress, lead to cell membrane damage, and result in ion leakage from the membrane (Zhao *et al.* 2008). Nile red staining and ion leakage measurements were performed to determine possible membrane injuries. Observation of cells stained with Nile red (Fig. 6) indicated that with *A. nodosum* extract treatment (Fig. 6*c*, *d*), the cells exhibited minor membrane disruption and damage. However, the cells in the control (Fig. 6*a*, *b*) displayed a higher rate of membrane damage; they also showed a higher percentage of leakage (*diamond-marked line*; Fig. 7) and a greater number of membrane droplets (Fig. 6*a*). Cultures supplemented with 0.1 g L^{-1} of *A. nodosum* extract showed the lowest ion leakage, ranging from 5% during the − 5°C cold treatment to 37% after recovery at day 7 (*x-marked line*; Fig. 7), indicating the maximum protection under stress. On the other hand, leakage was recorded as 53% for the 0.05 g L^{-1} *A. nodosum* treatment (*triangle-marked line*; Fig. 7), and 65% for both the control and the 0.01 g L^{-1} treatment (*diamond-marked line* and *square-marked line*, respectively; Fig. 7), after recovery.

Cell staining with the fluorescent dye DAPI, which binds to DNA, showed that the treatment with the *A. nodosum* extract

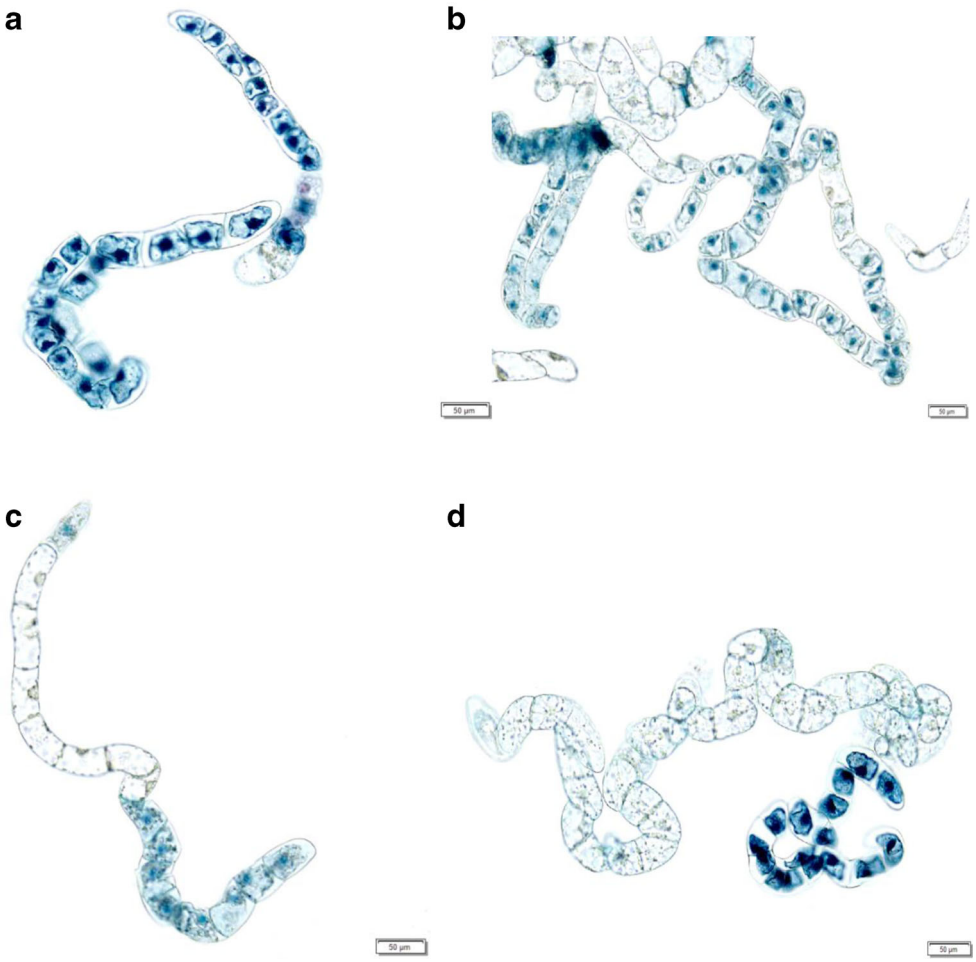

Figure 5. Effect of *Ascophyllum nodosum* extract on *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) cell viability under freezing stress, determined by Trypan blue exclusion staining. The viability of cells supplemented with (*a*) 0, (*b*) 0.01, (*c*) 0.05, and (*d*) 0.1 g L^{-1} of seaweed extract at − 5°C was assessed. The cells stained blue are dead cells. *Scale bars*, 50 μm.

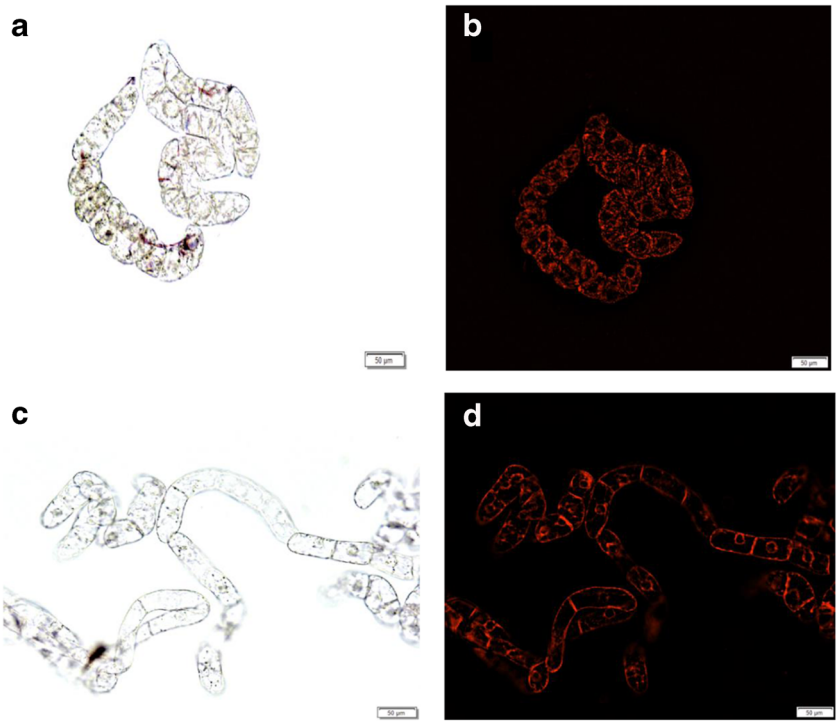

Figure 6. Effect of *Ascophyllum nodosum* extract on *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) cell membranes under freezing stress at − 5°C, determined by Nile red staining. Membrane damage in cells without seaweed extract treatment after freezing stress, visualized by (*a*) bright field and (*b*) fluorescent images. Membrane damage of cells treated with seaweed extract after freezing stress, (*c*) bright field, and (*d*) fluorescent microscopy (excitation 450–500 nm and emission 529 nm). *Scale bars*, 50 μm.

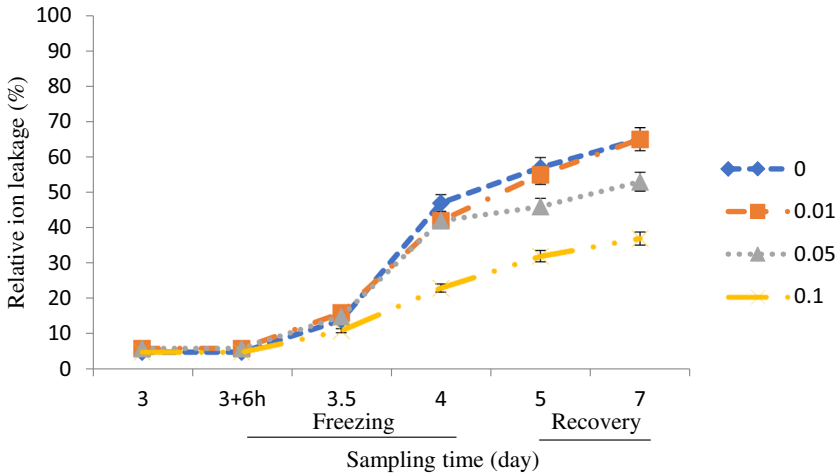

Figure 7. Ion leakage changes in cold-stressed *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) suspension cultures treated with *Ascophyllum nodosum* extract. The cells were treated with various concentrations of seaweed extract, 0, *diamond-marked line*; 0.01, *square-marked line*; 0.05, *triangle-marked line*; and 0.1 g L^{-1}, *x-marked line*; and the ion leakage was determined at time intervals before, during, and after freezing stress of − 5°C. The *error bars* represent standard deviation.

(Fig. 8*c*, *d*) resulted in protection of the nucleus at − 5°C. The microscopy studies showed that the nuclei were large and circular in shape compared to untreated control cultures (Fig. 8*a*, *b*). Untreated cells displayed unevenly shaped nuclei, which were not located at the center of cells, but rather along the sides (Fig. 8*a*, *b*).

Gene expression analysis of *A. nodosum* extract-treated cells under freezing stress In order to determine whether the induction of seaweed extract increase in cell growth or protection induced by *A. nodosum* extract in BY-2 cultures under freezing stress was due to an active mechanism requiring gene expression regulation, cells under − 5°C freezing stress, without (control) and treated with 0.1 g L^{-1} of *A. nodosum* extract, were used for gene expression analysis by qRT-PCR.

The results showed that *fucosyltransferase* indicated the maximum expression (6.8-fold), in untreated cultures after stress (Fig. 9*a*). The transcript level of *betaine aldehyde dehydrogenase-1* (*BADH1*) reached a 10-fold maximum in control cultures during the recovery period (Fig. 9*b*). The maximum induction for *galactinol synthase 2* (Fig. 9*c*) was recorded as 5.13-fold, which was seen on day 4 during freezing of *A. nodosum* extract-treated cells. Interestingly, *galactinol synthase 2* was not detected in the cultures before freezing (Fig. 9*c*). The peak induction of *glutathione S-transferase* was 37.1-fold (Fig. 9*d*), and that of the *activating protein-2*

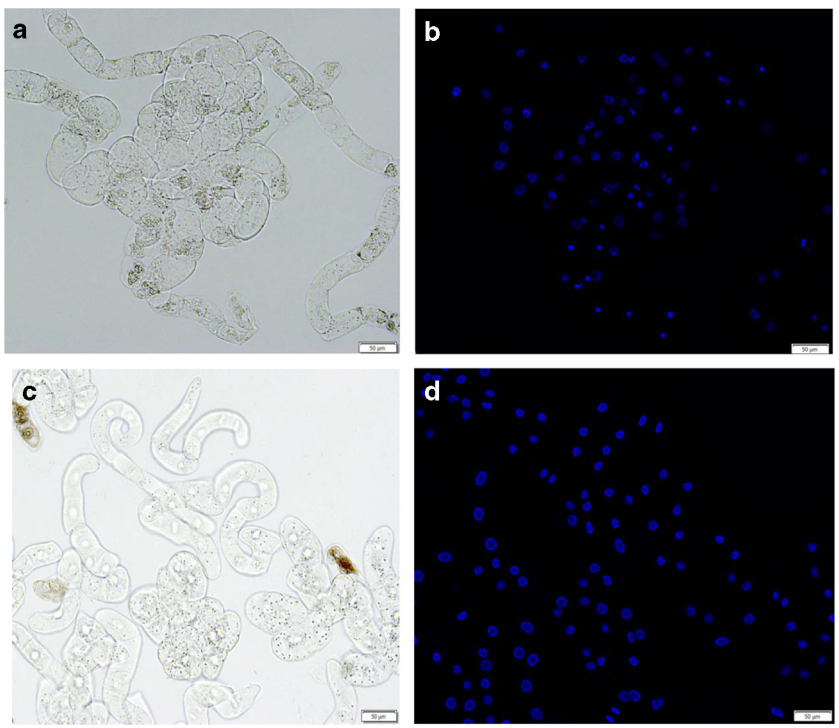

Figure 8. Effect of 0.1 g L^{-1} *Ascophyllum nodosum* extract on *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) cell nuclei under freezing stress at − 5°C, visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. Freezing induced chromatin damage in cells without seaweed extract treatment, as seen in (*a*) bright field and (*b*) fluorescent images; (*c*) bright field and (*d*) fluorescent microscopy of BY-2 cells treated with seaweed extract after freezing stress (excitation 359 nm and emission 461 nm). *Scale bars*, 50 μm.

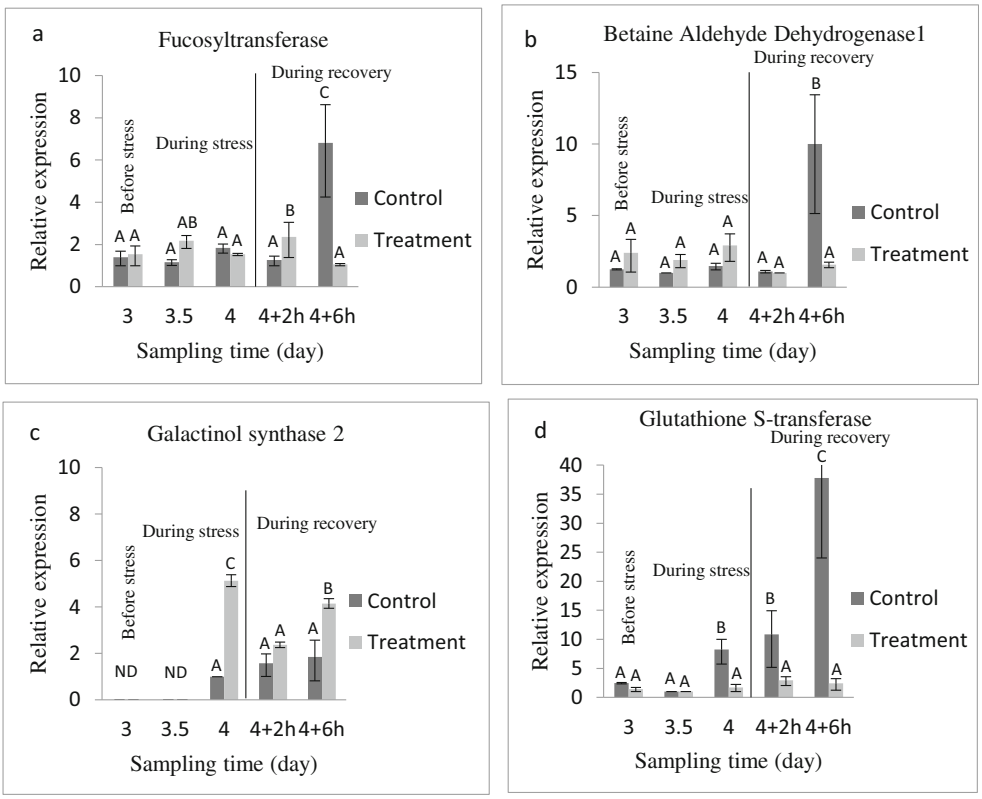

Figure 9. Differential expression patterns of selected genes in *Ascophyllum nodosum*-extract treated (*treatment*) and untreated (*control*) *Nicotiana tabacum* cv Bright Yellow-2 (BY-2) cells, before, during, and after exposure to freezing temperature of − 5°C. (*a*) *Fucosyltransferase*, (*b*) *betaine aldehyde dehydrogenase 1*, (*c*) *galactinol synthase 2*, (*d*) *glutathione S-transferase*, (*e*) *activating protein 2*, (*f*) *pyrroline-5-carboxylate synthase*, (*g*) *acetyl-CoA carboxylase*, and (*h*) *digalactosyldiacylglycerol synthase*. *Error bars* indicate standard deviation. *Different letters* represent significant difference among the concentrations of *A. nodosum* extract at $\alpha < 0.05$ by Tukey test. *ND*, not detected.

(*AP2*) was 9.06-fold (Fig. 9*e*), for recovered control cells after freezing stress. *Pyrroline-5-carboxylate synthase* showed the highest expression as a 7.03-fold increase in the control cells at recovery stage (Fig. 9*f*). *Acetyl-CoA carboxylase* produced

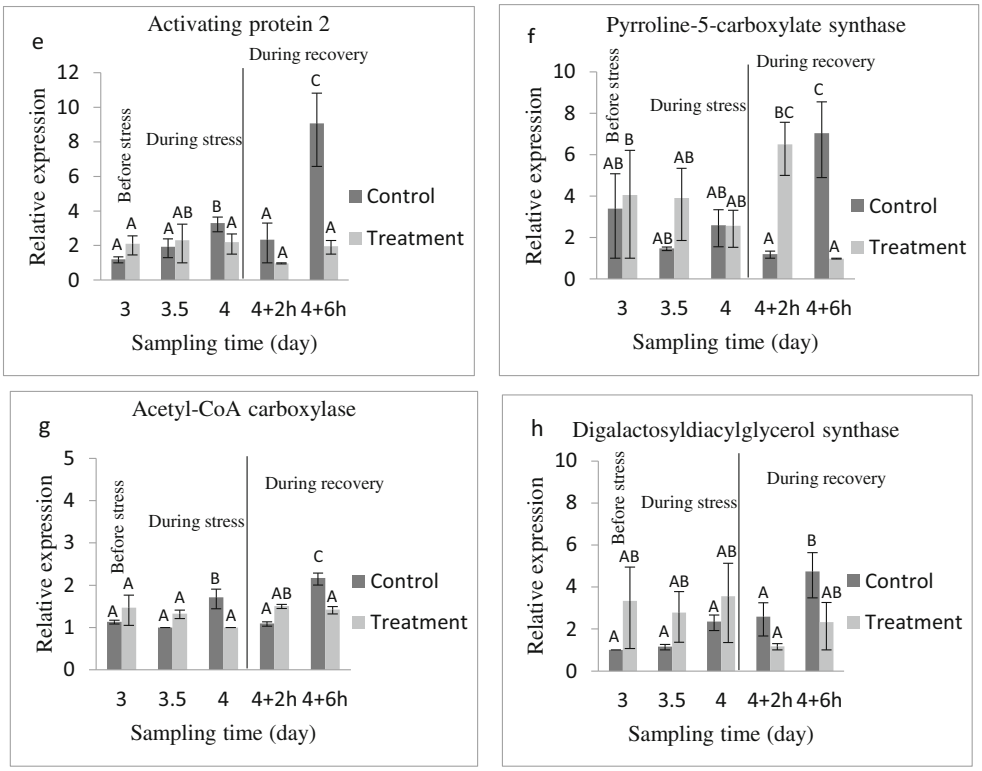

Fig. 9 (continued)

a peak induction of 2.17-fold after stress and in untreated cultures (Fig. 9*g*), and the gene expression level of *Digalactosyldiacylglycerol synthase* (*DGD1*) reached 4.73-fold increase in untreated cultures (Fig. 9*h*).

The current study found that *A. nodosum* extract treatment resulted in decreased cell growth in the absence of cold stress. This observation corroborated earlier reports where sulfated fucans isolated from *A. nodosum* cell walls caused antiproliferative responses in a fibroblast tumor cell line (Haroun-Bouhedja *et al.* 2000). In this present study, the *A. nodosum* treatment was used on BY-2 suspension cells; however, plants are far more complex systems and may react differently even under identical stress conditions. Previous reports of the use of algal extract treatments used on the same plant cultivar have not been found. Therefore, it is to be expected that *A. nodosum* extract may elicit a different response in another species or in whole plants. However, as the current investigation demonstrated, although the *A. nodosum* extract did not stimulate growth at 27°C, it offered resistance against freezing stress, helped cells survive after stress, and led to a higher dry weight under freezing temperatures. Previously, it was demonstrated that algal extract contained different nutrient components such as sugars, organic materials, and minerals, which may play a role in various effects of this extract (Rayirath *et al.* 2009; Battacharyya *et al.* 2015).

Ascophyllum nodosum extract treatment of BY-2 cells undergoing freeze stress caused differential expression of some genes and activated different cellular mechanisms. *Activating protein 2* (AP2 transcription factor) has been shown to function as a cold stress responsive gene (Du *et al.* 2016). Rayirath *et al.* (2009) showed that following algal extract treatment of *Arabidopsis thaliana* (L.) Heynh. at freezing temperatures, downregulation of *AP2* occurred. In another study conducted by Du *et al.* (2016) on *Brassica napus* L. (rapeseed) under cold stress, *AP2* gene expression was delayed until 2 h after starting the stress, and the transcript level reached maximum 12 h after the induction of cold stress. In the current study, *AP2* showed a reduced expression in *A. nodosum* extract-treated cultures after the stress and during recovery. Under the same condition, *AP2* expression was nine times higher in the untreated control, showing that those cultures were experiencing a more difficult stress condition than cells with the extract.

Cells experiencing such stress conditions undergo structural changes, such as deformation of the nucleus and cell dehydration, resulting in cell death (Van Breusegem and Dat 2006). Applying algal extract to salt-stressed tobacco BY-2 cultures or cold-stressed *Arabidopsis* prevented the formation of dehydrated cells and reduced deformation of nuclei, whereas the cultures without algal extract treatment appeared to have a greater number of abnormally shaped nuclei (Banu *et al.* 2009; Rayirath *et al.* 2009). The present study showed that treatment with *A. nodosum* extract reduced cell damage and death after freezing treatment. The recovery period after stress, during which untreated cells showed the maximum percentage of cell death, was extremely crucial for cell survival. It was supposed that the osmo-protectant activity of the treatment helped cells adjust their osmotic pressure and overcome the rapid change of temperature. In other words, returning the freezing stressed cells to 27°C caused an imbalanced osmotic pressure in the controls, in contrast to the treated cells, where the extract appeared to prevent this disruption of cellular osmotic balance. Therefore, the lowered expression of certain cold stress genes, especially *AP2*, might indicate that cells treated with *A. nodosum* extract had less difficulty in dealing with the stress at the cellular level.

Furthermore, accumulation of organic materials, such as betaines and proline, may occur under stress. During periods of dehydration, plant cells increase their betaine content (Kishitani *et al.* 1994; Ashraf and Foolad 2007). *Betaine aldehyde dehydrogenase* (*BADH*) is involved in betaine biosynthesis (Kishitani *et al.* 1994). Additionally, algal extract contains betaines, which acts as antifreeze (Rayirath *et al.* 2009). The differential expression of genes related to accumulation of such compounds also affects the stress tolerance in host plants. In the present study, the *A. nodosum* extract treatment reduced *BADH* expression in cultured cells during freezing stress which could be due to the stress-protective effect of algal extract on the cells. Additionally, it should be noted that the accumulation of proline might not be increased by increasing its gene expression under stress. *Pyrroline5-carboxylate synthase* (*P5CS*), which is involved in proline biosynthesis (Dombrowski *et al.* 2008) showed as a time-dependent factor in *Solanum lycopersicum* L. (tomato) plants under drought stress (Karunatilleke 2014), where irrigation decreased *P5CS* expression levels in all plants regardless of the treatments. In the current study, it was observed that *P5CS* transcript showed higher expression after stress, within 2 h or 6 h post cold stress in the *A. nodosum*-treated or control groups, respectively (Fig. 9*f*).

The antioxidant-inducing activity of the algal extract may play a role in cell protection under stress (Rayirath *et al.* 2009; Nair *et al.* 2012). Cell and organelle functions under stress are damaged due to build-up of reactive oxygen species, which are known to induce apoptosis (de Pinto *et al.* 2002) and might be reduced in the extract-treated cells. The extract includes phenolic compounds, and their beneficial effects as scavengers of reactive oxygen species are well documented (Zhang and Ervin 2008; Nair *et al.* 2012). Lower production of free radicals leads to lower incidence of PCD and thus higher cell viability (Van Breusegem and Dat 2006). Studies have reported that algal extract increases phenolic and antioxidants compounds in *Spinacia oleracea* L. (spinach), which when fed to *Ceanorhabditis elegans*, improved their oxidative stress and heat tolerance (Fan *et al.* 2011; Battacharyya *et al.* 2015). Application of algal extract also increased the antioxidant activity and high temperature tolerance in *Agrostis stolonifera* L.

(creeping bentgrass; Zhang and Ervin 2008). The upregulation of antioxidant coding genes in *Nicotiana tabacum* increases the plant's tolerance to abiotic stresses (Le Martret *et al.* 2011).

In the current study, the relative expression of *glutathione S-transferase* (*GST*) was significantly lower in cells treated with the *A. nodosum* extract, which was in line with another report by Karunatilleke (2014), where tomato plants showed a lowered activity in antioxidant enzymes under water stress; despite that, they were more tolerant to the stress compared to the plants without extract treatment. The low expression of the *GST* in cultures under the extract treatment observed in the current report was probably due to lower oxidative and stress damage, as discussed earlier.

Moreover, it has been reported that sugars play a central role in the modulation of oxidative stress in plants, as sugars coordinate cell responses to environmental factors by activating pathways and regulating genes (Couée *et al.* 2006). The algal extract contains different and complex sugars, which may be involved in stress regulation. The expression of galactinol synthase, involved in synthesis of raffinose, is associated with environmental stress (Nishizawa *et al.* 2008). Treatment with lipophilic components of algal extract increased galactinol synthase in *A. thaliana* under freezing (Rayirath *et al.* 2009). In the present study, *galactinol synthase 2* was upregulated under freezing stress in the extract-treated group, which might also indicate that the influence of *A. nodosum* extract on raffinose production had a part in stress tolerance.

The plasma membrane is a major site to suffer from various abiotic stresses and it is possible that algal extract changes membrane characteristics, thus enhancing plant tolerance to freezing stress (Rayirath *et al.* 2009). In the current report, staining with Nile red displayed numerous spherical bodies in control cells, compared to those treated with *A. nodosum* extract. As the indicator of cell membrane damage, similar lipid droplets have been observed under temperature stress (Greenspan *et al.* 1985). In response to freezing, digalactosyldiacylglycerol (DGDG) is produced in cell membranes. The enzyme DGDG synthase (DGD1) that functions in DGDG production is active in the production of cell membrane lipids, sub-cellular lipid trafficking, growth, and development (Li *et al.* 2008; Degenkolbe *et al.* 2012). It has been reported that algal extract upregulates the *DGD1* gene under freezing stress and thus improves the stability of cell membrane in *A. thaliana* (Rayirath *et al.* 2009; Nair *et al.* 2012). In the present study, under treatment of *A. nodosum* extract, *DGD1* did not show higher expression levels after stress, but rather was consistently elevated in the other stages. This may underscore the importance of the extract as an osmo-protectant. On the other hand, the higher expression of *DGD1* in the controls recovering after stress might represent a final effort of cells to repair membrane damage in order to survive, which was ultimately unsuccessful for most of the control cells. Other factors, for instance, acetyl-CoA carboxylase (ACCase), associated with production of fatty acids, increase the membrane stability (Sasaki and Nagano 2004; Xiao and Chye 2011). The suggested role of algal extract in maintaining membrane integrity under freezing stress encouraged Khan *et al.* (2009) to elucidate the expression of genes involved in this process. The analysis of *Acetyl-CoA* gene expression presented in the current report revealed an early induction in cultures under the extract treatment, which could be interpreted as a stress responsive mechanism for recovering plant cells to take, in order to decrease membrane damage received during the stress.

Apart from oxidative protection, osmo-protectant activity, membrane stability, and differential gene expression roles within cells exposed to *A. nodosum* extract, enzymes active in cell wall biosynthesis may influence cell integrity. Fucosyltransferase is one of these enzymes that was expressed in *A. thaliana* cultures experiencing sub-zero temperature stress while under algal extract treatment (Rayirath *et al.* 2009). Tobacco BY-2 cells lack the fucosyl form of this enzyme (Wu *et al.* 2010), which might explain the low expression of this gene observed in the current study. However, to uncover and confirm the role of algal extract in cell wall synthesis, further analysis should be conducted. It is likely that a combination of the abovementioned mechanisms was induced by the application of *A. nodosum* extract and led to BY-2 cell protection under freezing stress.

Conclusion

In conclusion, this study indicated that BY-2 cells provided an appropriate model system to evaluate the effects of *A. nodosum* extract on the survival of cells exposed to freezing temperatures. The current study clearly showed the cold protective role of *A. nodosum* extract treatment in BY-2 cells, whereby cells could survive − 5°C temperature, whereas the viability of controls decreased significantly. Obviously, differential expression of multiple genes and activation of repair mechanisms were involved, in order to protect cells from such severe environmental conditions. The antifreeze function of *A. nodosum* extract makes this natural material a valuable product for the study of the detailed mechanisms involved. Further experiments to reveal the precise mechanism of *A. nodosum* extract action remain to be conducted. It is believed that the current study constitutes the first attempt of studying the protective effects of *A. nodosum* extract on tobacco BY-2 cell cultures experiencing cold stress. The complexity of environmental factors and heterogeneity of the plant response towards stress, the algal extract imparts abiotic stress tolerance in different plant species to a different extent. Moreover, it should be acknowledged that even within the same species, the behavior of plants can vary from that of cell

cultures, and a complexity of experimental outcomes may result from applying the current findings to whole plants. However, the basic mechanisms of responses to cold stress should be the same between cells and plants. The present study, along with other reports, suggested that the antioxidant activity of algal extract was a very important mechanism by which algal extract mitigated plant responses to environmental stress. Although several studies have addressed this topic and have identified the activity of different antioxidant enzymes under different stress and algal extract treatments, not all antioxidants have been measured. Future studies are warranted to identify the underlying trends of *A. nodosum* extract antioxidant activity and may further focus on whether *A. nodosum* extract can prevent PCD in cells under temperature stress. Studies examining both, the antioxidant activity of *A. nodosum* extract in combination with PCD, may shed light on this important resistance mechanism. The cryoprotective aspects of *A. nodosum* extract may ultimately be beneficial for plant growers to take advantage of a natural substance and increase the productivity of farms.

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Authors' contributions BP designed and supervised the research. MZ conducted experiments and wrote the manuscript. AC and JN contributed in providing resources, supporting the research, and advising throughout the experiments. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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