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Regulation of Phytohormone Biosynthesis and Accumulation in *Arabidopsis* Following Treatment with Commercial Extract from the Marine Macroalga *Ascophyllum nodosum*

Owen S. D. Wally · Alan T. Critchley · David Hiltz · James S. Craigie · Xiumei Han · L. Irina Zaharia · Suzanne R. Abrams · Balakrishnan Prithiviraj

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Abstract Seaweeds and their extracts have been used for centuries in agriculture to improve plant growth and impart stress tolerance. There has been historical evidence that phytohormones present in seaweeds lead to these effects, but questions of this mode of action have always been raised. By quantifying phytohormones in seaweed extracts coupled with the use of phytohormone biosynthetic and insensitive mutants, we conclude that the phytohormone levels present within the extracts are insufficient to cause significant effects in plants when extracts are applied at recommended rates. However, components within seaweed extracts may modulate innate pathways for the biosynthesis of phytohormones in plants. Phytohormone profiles of plant tissue extracts were analyzed following root application of a commercial seaweed extract produced from *Ascophyllum nodosum* (ANE) to in vitro-grown *Arabidopsis* plants. We found an increase in total concentration of cytokinins (CKs), in particular, of *trans*-zeatin-type CKs, 24 and 96 h after ANE application, with an increase in *cis*-zeatin-type CKs observed at 144 h. Concomitantly, increases in abscisic acid (ABA) and ABA catabolite levels were observed whereas auxin levels were reduced. Additionally, the profile of transcripts revealed that CK biosynthetic genes were upregulated, whereas the CK catabolic genes were repressed at 24 and 96 h following ANE application. Not surprisingly, the transcripts of ABA biosynthetic genes were increased whereas the auxin biosynthetic genes were repressed. These corroborated findings are the first to help explain the underlying physiological benefits derived from the application of ANE to plants.

Keywords *Ascophyllum nodosum* · Abscisic acid · *Arabidopsis* · Auxins · Cytokinins · Gene expression · Phytohormones

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O. S. D. Wally
Department of Environmental Sciences, Nova Scotia Agriculture College, Truro, NS B2N 5E3, Canada

A. T. Critchley · D. Hiltz
Acadian Seaplants Limited, Dartmouth, NS B3B 1X8, Canada

J. S. Craigie
Institute for Marine Biosciences, National Research Council of Canada, Halifax, NS B3H 3Z1, Canada

X. Han · L. I. Zaharia · S. R. Abrams
Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK S7N 0W9, Canada

B. Prithiviraj (✉)
Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, NS B2N 5E3, Canada
e-mail: bprithiviraj@dal.ca

Introduction

Ascophyllum nodosum, a brown macroalga known as rockweed, is ubiquitous in cool coastal marine waters throughout the Northern Hemisphere. Unprocessed *A. nodosum* and alkaline extracts derived from the alga have been widely used as a biostimulant in agricultural production in a wide variety of crop species (Craigie 2011; MacKinnon and others 2010; Spann and Little 2011). Direct benefits from the application of *A. nodosum* and other seaweed extracts on crop performance include enhanced root vigor (Crouch and van Staden 1992), increased leaf chlorophyll content (Blunden and others 1996), an increase in the number of leaves (Rayirath and others 2008), improved fruit yield (Arthur and others 2003; Kumar and Sahoo 2011; Kumari and others 2011),

heightened flavonoid content (Fan and others 2011), and enhanced vegetation propagation (Leclerc and others 2006). However, the more substantial improvements associated with application of seaweed extracts are improved tolerance toward abiotic stresses, including drought (Spann and Little 2011; Zhang and Ervin 2004), ion toxicity (Mancuso and others 2006), freezing (Rayirath and others 2009), and high temperature (Zhang and Ervin 2008).

The precise mechanisms by which seaweed extracts improve the growth and vigor of plants are not fully understood, but many of the extracts' effects are attributed to a variety of constituents within the seaweed extracts, including betaines, plant nutrients (both micro and macro), and phytohormones (Khan and others 2009). Although the modes of action of some of the chemical components of seaweed extracts are known, the vast majority remain uncharacterized. Therefore, it is likely that many of these biochemical components within the extracts exhibit additive or synergistic activities (Fornes and others 2002). The chemical compositions of several seaweed extracts are known, and because they can maintain plant-promoting bioactivity at relatively low concentrations (<0.01 % w/v) (Crouch and van Staden 1993) or in the absence of ionic compounds (Rayirath and others 2008), it is unlikely that the growth-promoting ability is due to nutrient composition alone. Instead, the beneficial effects of seaweed extracts appear to be modulated through either plant growth regulators within the seaweed extracts or through a process of stimulation of the endogenous phytohormone biosynthesis in extract-treated plants.

Phytohormones are low-molecular-weight natural products produced by plants. They essentially regulate all physiological and developmental processes from germination to senescence as well as plant responses to environmental stresses. These structurally diverse compounds include auxins, cytokinins (CK), abscisic acid (ABA), gibberellic acid (GA), ethylene, jasmonic acid, and salicylic acid. Several biosynthetic precursors and catabolic metabolites of these plant hormones can exhibit biological activity, giving rise to an intricate network of signalling molecules at the cellular level. To further complicate the picture, there is mounting evidence of considerable cross-talk among plant hormone-signaling pathways in regulating developmental and physiological processes (Peleg and Blumwald 2011; Su and others 2011; Werner and Schmulling 2009).

Based on phenotypic observations of plants treated with various seaweed extracts, CK and cytokinin-like activity is the most commonly reported phytohormone effect of seaweed extracts (Stirk and others 2003). CKs are among the most important plant growth regulators, controlling plant cell division, the release of axillary buds, and induction of photomorphogenic development, in addition to being the dominant signal that controls growth rates and development within the shoot apical meristem (Werner and Schmulling 2009). In *Arabidopsis*, *trans*-CKs are considered to be more active than *cis*-CKs, with *trans*-zeatin (*t*Z) and isopentyl adenine (2iP) types being dominant in controlling CK-dependent responses (Sakakibara 2006a, b). The initial step in the biosynthesis of *trans*-CKs in *Arabidopsis* is through the formation of iP-ribotides from adenosine subunits via the action of isopentenyl transferases (AtIPT1,3-8) (Miyawaki and others 2004, 2006). CK signalling follows a multi-component phosphorylation cascade. Initially, CKs are received by His protein kinases [*Arabidopsis* histidine kinases (AHK) 2, 3, and 4], which then phosphorylate His phosphotransfer proteins [*Arabidopsis* histidine phosphotransfer proteins (AHPs)], which are then transferred to the nucleus and phosphorylate *Arabidopsis* response regulators (ARRs) (Argueso and others 2010; D'Agostino and others 2000; Kieber and Schaller 2010; Perilli and others 2010; To and others 2007; Werner and Schmulling 2009). Phosphorylated ARRs act as transcription factors inducing the transcription of CK responsive genes (Perilli and others 2010). Degradation of CKs is controlled mainly through the action of cytokinin oxidase/dehydrogenase (CKX) which is encoded by a multigene family in *Arabidopsis* (*AtCKX1-7*) (Kudo and others 2010; Werner and others 2006).

Application of exogenous CKs at low levels resulted in increased leaf size, increased seed set, and delayed senescence while resulting in overall reduction in root mass (Nooden and others 1979; Singh and others 1992). Overexpression of various *IPT* genes under the control of induced or developmentally regulated genes resulted in plants that exhibited an increase in compound leaf numbers, leaf area, chlorophyll levels and seed set, while the plants exhibited decreased root mass and a delay in leaf senescence (Gan and Amasino 1995; Ghanem and others 2011; Rivero and others 2007, 2010; Shani and others 2010). In addition to improved above-ground plant growth, many of these enhanced CK-producing plants also had improved tolerance against salinity and drought stress (Havlova and others 2008; Merewitz and others 2011; Rivero and others 2007). In contrast, plants with mutations in *IPT* genes or that overexpressed *CKX* had reduced CK content leading to plants with reduced leaf area, decreased shoot formation, and an overall increase in root mass (Matsumoto-Kitano and others 2008; Shani and others 2010; Werner and others 2001, 2003, 2010).

In addition to CK-like effects observed in plants treated with seaweed extracts, seaweeds themselves, their extracts, and seaweed-treated plants have been reported to contain high levels of auxins and auxin-like compounds (Crouch and van Staden 1993; Kumari and others 2011; Yokoya and others 2010). Auxins control the rate of cellular expansion through the action of auxin response factors (ARF) that perceive the auxin translocated through the

action of Pin-formed (PIN) proteins and activate auxin responsive genes (Bishopp and others 2011; Moubayidin and others 2009). Auxin biosynthesis is complex, with five biosynthetic pathways described in *Arabidopsis* [4 tryptophan (trp)-dependent and one trp-independent (Zhao 2010)]. A commercial extract of *A. nodosum* had an estimated concentration as high as 50 mg g^{-1} dry weight (DW) of indole acetic acid (IAA) (Kingman and Moore 1982). Auxin-like responses have been observed through proliferation of adventitious roots in cut mung bean shoots immersed in a variety of aqueous seaweed extracts (Kumari and others 2011). In addition, the organic components of *A. nodosum* extracts were shown to activate the GUS expression driven by the synthetic auxin responsive promoter DR5 in transgenic *Arabidopsis* plants (Rayirath and others 2008).

In the present study, we attempted to identify the mechanisms by which application of the alkaline ANE alters phytohormone levels in plants by quantifying changes in phytohormone concentration and transcript abundance of phytohormone metabolic genes.

Materials and Methods

Plant Growth

Arabidopsis thaliana (L. Heyhn.) plants, ecotype Columbia (Col-0), were grown in in vitro culture. Seeds were first gas sterilized with NaClO (5 % v/v) and concentrated HCl (0.3 % v/v) for 3 h, suspended in 0.15 % agar (Bioshop Canada, Inc., Burlington, ON, Canada), and stored at 4 °C in the dark. After 2–3 days of stratification, seeds were sown on sterilized filter paper within a 100-ml glass jar containing 10 ml of ½ MS medium (Murashige and Skoog salt, Sigma, St. Louis, MO, USA) supplemented with 1 % (w/v) sucrose pH 5.7, which was sealed with a Magenta B cap (Sigma). The seeds were then grown in a growth chamber at 22 °C with a 16-h light/8-h dark cycle with a light intensity of 100 μmol m^{-2} s^{-1}.

Chemical Treatment

Soluble seaweed extract powder (ANE), commercially produced from *A. nodosum* (Acadian Seaplants Limited, Dartmouth, NS, Canada), was used in the experiment. The control treatment was a modified Long Ashton Nutrient Solution (LANS) that contained inorganic ions that were present in the ANE [1.7 mM KOH, 1.65 mM KCl, 400 μM KNO_3, 400 μM $Ca(NO_3)_2$, 150 μM $MgSO_4$, 133 μM NaH_2PO_4, 10 μM NaCl, 5 μM $C_6H_5FeO_7$, 3 μM H_3BO_3, 1 μM $MnSO_4$, 500 nM $CuSO_4$, 200 nM $ZnSO_4$, 50 nM Na_2MoO_4, and 20 nM $CoSO_4$]. Both test solutions were adjusted to pH 5.7 and filter sterilized before being added to 14-day-old plants at a final rate of 0.01 % w/v.

Phytohormone Analysis

Rosette leaves from 25 *Arabidopsis* plants were harvested [~ 1 g fresh weight (FW)] at 24, 96, and 144 h following ANE treatment. There were three biological replicates for each treatment and time point. The whole experiment was repeated twice over a period of 4 months. Samples were flash frozen and lyophilized, precisely weighed, and processed according to Chiwocha and others (2003). Seaweed extracts of powder and liquid origins were lyophilized, with 1 g used for analysis. Quantification of ABA, cytokinins, auxins, and GAs in control and ANE-treated plant tissue was conducted using ultraperformance liquid chromatography–electrospray tandem mass spectrometry (UPLC-ESI–MS/MS: http://www.nrc-cnrc.gc.ca/eng/facilities/pbi/plant-hormone.html). Particulate matter and other unwanted components were removed by solid-phase extraction (SPE) with C18 SepPak cartridges (Waters, Mississauga, ON, Canada). The procedure used for quantification of endogenous auxins, abscisic acid, and metabolites GA and cytokinins was performed as described in Chiwocha and others (2003). Internal standards used for analysis were synthesized or obtained as described previously (Abrams and others 2003; Chiwocha and others 2003, 2005; Zaharia and others 2005). Calibration curves were created for all compounds of interest. Quality control samples (QCs) were run along with the tissue samples. Detailed methods for phytohormone analysis are described in Supplementary Methods.

Root Growth Assay

In addition to wild-type seeds, the following *Arabidopsis thaliana* (Columbia ecotype Col-0) plants were obtained from the Arabidopsis Stock Center in Columbus, OH, USA: *abi4-1* mutants, *DR5:GUS*, and the quadruple mutant *ipt1,3,5,7* donated by Dr. Tatsuo Kakimoto (University of Osaka) (Matsumoto-Kitano and others 2008) and the *ARR5::GUS* in the Wassilewskija (WS-0) background. Gas-sterilized *Arabidopsis* seeds (wild-type Col-0; abi4-1, DR5:GUS, ARR5::GUS, and ipt1,3,5,7) were spread onto ½ MS media and stratified at 4 °C for 3 days before being oriented vertically in the growth chamber. Evenly germinated seedlings were transferred after 4 days to square ½ MS plates supplemented with the different concentrations of ANE. Root length was measured on 7-, 9-, and 11-day-old seedlings, and relative root growth rates were evaluated by comparison with the control using ImageJ software (Research Services Branch, NIH, Bethesda, MD, USA).

Lateral Root Quantification

The number of lateral roots were counted on transferred seedlings starting 7 days post germination and then grown on ½ MS medium containing ANE or control. The number of lateral roots on the primary root was determined with a dissecting microscope; all lateral roots that had emerged from the primary root were counted.

For root growth and GUS assays, gas-sterilized *Arabidopsis* seeds were plated onto ½ MS media solidified with 0.25 % (w/v) Phytagel (Sigma), stratified for 3 days at 4 °C, and then transferred vertically to growth chamber conditions. Four days after germination the seedlings were transferred to square plates containing solidified ½ MS media supplemented with different treatments.

GUS Staining

Plant tissues were fixed in 90 % (v/v) acetone for GUS staining, which was performed as described previously (Weigel and Glazebrook 2002). Pictures were taken with a Canon digital camera (EOS Rebel) using a stereoscope microscope. Each treatment was performed using a minimum of three biological replicates.

qRT-PCR Analysis

Total RNA was isolated using the monophasic RNA extraction method (Chomczynski and Sacchi 1987). Two micrograms of RNA was treated with 2 units of RQ1 DNAse (Promega, Madison, WI, USA) according to the manufacturer's instructions. cDNA was synthesized using an Applied Biosystems high-capacity cDNA synthesis kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), using the manufacturer's protocols. Relative transcript levels were assayed by real-time PCR analysis using gene-specific primers on a StepOne™ Plus Real-Time PCR System (Applied Biosystems), which used the Power SYBR® Green RT-PCR Reagents kit (Applied Biosystems). Primer sequences were taken from the Roche Universal Probe Library (Roche Diagnostics, Indianapolis, IN, USA). Wherever possible, the primers would flank an intron-spanning region. All amplicon-length ranges were between 68 and 120 bp. The reaction mixture contained 5 µL of Power SYBR® Green PCR Master Mix, 20 ng of cDNA, and 250 nM of each of the forward and reverse primers. The following default program was used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min each. RNA relative quantification analyses were performed using *Actin2* serving as an endogenous control, with efficiencies calculated by dilution series for individual primers (Pfaffl 2001). The list of primers used is shown in Supplementary Table 2. Each data point was determined in triplicate for each of the three biological replicates and presented as mean ± SE.

Statistical Analysis

For all analyses, ANE-treated plants were compared to control-treated plants, with the differences analyzed using analysis-of-variance Tukey's honestly significant difference test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) with SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Phytohormone Analyses of *A. nodosum* Extracts

To determine if previously reported increases in "phytohormone-like"responses were due to phytohormones present within seaweed extracts themselves, we quantified the phytohormones in various seaweed extracts. Total phytohormone levels were quantified in *A. nodosum* alkaline extracts from Canadian Atlantic sources used in this study, in addition to various other commercially available extracts (Table 1). The phytohormone levels in all of the extracts were relatively low, with isopentenyl adenine (2iP) being the only CK detected in all of the samples at levels close to 10 ng g^{-1} DW of sample. Similarly, the concentrations of ABA and its catabolites and all of the GAs were less than 5 ng g^{-1} DW. The Norwegian ANE had levels of IAA in excess of 500 ng g^{-1} DW; however, the remaining samples had lower levels in the 20–25-ng g^{-1} DW range, which would be unlikely to induce any auxin-responsive phenotypes at field application because minimum concentrations required for phenotypic changes are reported to be approximately 100 nM.

Effect of Seaweed Extracts on Rooting

Addition of aqueous ANE (0.01 % w/v) to ½ Murashige and Skoog (MS) media decreased the length of the primary roots at 3 and 5 days following treatment, a reduction of over 20 % compared to unsupplemented ½ MS medium (Fig. 1, $p < 0.01$). There was also a reduction in the total number of secondary roots, with ANE-treated plants having approximately 35 % fewer secondary roots per root primordia. Additionally, the ratio of secondary roots was reduced from 2.7 to 2.2 branches cm^{-1} ($p < 0.01$, data not shown). The overall morphology of the roots was also altered, with the secondary roots supplemented with *A. nodosum* extracts exhibiting reduced horizontal growth and growing in a more vertical fashion (Fig. 1a).

Both CKs and ABA are strong repressors of root growth in plants (Wasilewska and others 2008; Werner and

Table 1 Concentration of plant growth regulators in various commercial seaweed extracts (ng g^{-1} dry weight)

Seaweed extract	Auxins						ABA and ABA metabolites					Cytokinins				
	IAA	IAA-ala	IAA-asp	IAA-glu	IAA-leu	IBA	ABA	DPA	PA	*Neo*-PA	t-ABA	c-ZOG	t-Z	c-Z	2iP	iPA
Canadian Atlantic ANE 7/21/08	25	15	50	5	5	nd	2	nd	nd	nd	nd	5	nd	nd	10	5
Canadian Atlantic ANE 2/6/09	35	10	50	10	5	nd	1	nd	nd	nd	nd	5	nd	nd	10	5
Canadian Atlantic ANE 4/10/09	25	10	50	5	5	nd	1.5	nd	nd	nd	nd	5	nd	nd	10	5
French ANE	6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	nd
American Atlantic ANE	50	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	4.9
Irish ANE	nd	9.7	nd	nd	1.5	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.3	58.4
Norwegian ANE	615	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	10	45.7
S. African *Ecklonia* extract	nd	1	nd	1.5	nd	7.2	nd	nd	nd	nd	1	nd	nd	nd	nd	nd
S. African *Ecklonia* extract	21.2	1	nd	nd	nd	nd	1	nd	nd	nd	nd	nd	1	nd	1	1
N. Pacific *Macrocystis* extract	nd	1	nd	nd	1.1	12.5	1	nd	nd	nd	54	nd	nd	1.7	6.1	1.3
South Pacific *Durvilea*	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	27.7	1
Chinese *Sargassum* extract	nd	25	nd	nd	nd	1066	10	nd	nd	nd	nd	nd	10	nd	10	10

Approximately 1 g of seaweed extract from random samples was dried and pooled for analysis. Canadian Atlantic ANE harvested from three distinct batches were analyzed separately

IAA indole acetic acid, *IAA-ala* N-(indole-3-yl-acetyl)-alanine, *IAA-asp* N-(indole-3-yl-acetyl)-aspartic acid, IAA-glu = N-(indole-3-yl-acetyl)-glutamic acid, *IAA-leu* N-(indole-3-yl-acetyl)-leucine, *IBA* indole-3-butyric acid, *ABA cis*-abscisic acid, *DPA* dihydrophaseic acid, *PA* phaseic acid, *t-ABA trans*-ABA, *c-ZOG cis*-zeatin-O-glucoside, *t-Z trans*-zeatin, *2iP* isopentenyladenine, *iPA* isopentenyladenosine, *nd* not detected

Schmulling 2009). To determine if the observed reduction in root growth was due to the presence of either ABA or CKs within the ANE itself, we repeated the experiment with the ABA/CK-insensitive mutant *abi4-1* (Shkolnik-Inbar and Bar-Zvi 2010) and the CK biosynthesis quadruple mutant *ipt1,3,5,7* (Matsumoto-Kitano and others 2008). The *abi4-1* mutants exhibited decreased length of primary roots and increased lateral rooting ratios compared to Col-0 plants in both control and ANE treatment. However, the increased lateral root ratio was greater following ANE treatment with the *abi4-1* plants, which displayed a 50 % increase in lateral root number compared to only a 35 % increase with the control media (Supplementary Fig. 1). Additionally, there was no significant difference in primary root length in *abi4-1* plants grown on either control or ANE-supplemented media compared to the nearly 20 % reduction in primary root length in control Col-0 plants. When *ipt1,3,5,7* plants were grown on control medium, they produced thinner primary roots and a greater number of both secondary and adventitious roots, with the primary root similar in length to that of Col-0 plants (Supplementary Figs. 1 and 2). Similar to Col-0 plants, the mean length of primary roots for *ipt1,3,5,7* plants was also reduced. However, the length was reduced to a lesser extent than for Col-0 (Supplementary Figs. 1 and 2). The phenotype observed is possibly due to increased biosynthesis of endogenous phytohormones (that is, CKs and ABA) within the plant rather than an effect of the extract itself.

Effect on DR5 and ARR5 Reporters

Since we observed a decrease in root growth in the agar plate system following ANE treatment, we proceeded to determine if auxin and CK responses were altered using the synthetic auxin-responsive promoter *DR5* driving *GUS* genes in *Arabidopsis* Col-0, and the CK-sensitive promoter *ARR5* driving *GUS* expression in the WS-0 background of *Arabidopsis*. Forty-eight hours after transfer to media containing 0.01 % w/v ANE, GUS activity was greatly reduced throughout the *DR5*::*GUS* seedlings (Fig. 2). The activity in the controls was found throughout the vasculature of the cotyledons, with increasing intensity found at the root tips. The expression pattern was similar in plants transferred to media containing ANE, but with an overall lower intensity (Fig. 2). Five days after transfer the differences persisted. The GUS activity dissipated from the cotyledons of the control plants and was present within the vascular tissues of the fully formed leaves and relatively more intense within the newly emerging leaves (not

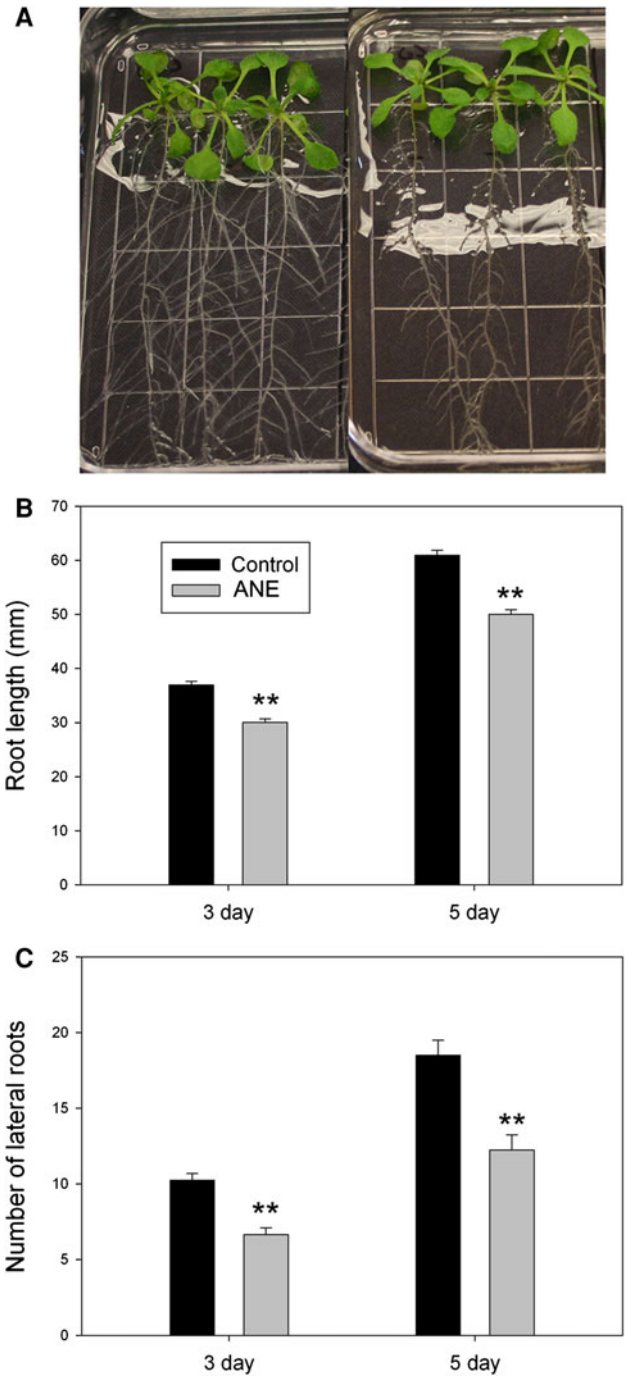

Fig. 1 Root growth and development was affected by ANE in agar plate system. **a** Twelve-day-old seedlings of wild-type *Arabidopsis*, 8 days after transfer to control (left) or ANE (0.01 % w/v) in ½ MS solid media. **b** Average length of primary roots in seedlings 3 and 5 days after transfer to indicated media. The data represent the mean ± SE ($n = 3$). **c** Number of lateral roots at all developmental stages (including lateral root primordial) in seedlings 3 and 5 days following transfer to media containing treatment

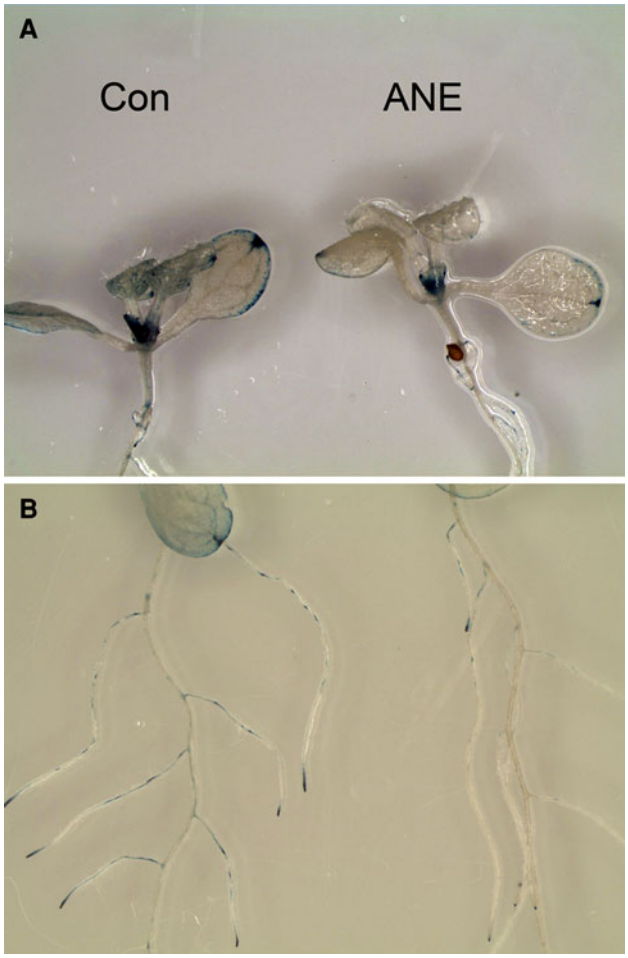

Fig. 2 ANE treatment alters auxin distribution in leaf tissues and roots. GUS staining of *DR5::GUS* transgenic seedlings 48 h after transfer to ½ MS control or ANE (0.01 % w/v) medium. **a** Cotyledons, emerging leaves, and shoot apical meristem. **b** Primary and lateral roots

shown). In contrast, leaves of the ANE-treated plants exhibited much lower levels of GUS activity, with nearly undetectable levels within the vasculature of fully formed leaves and relatively weak levels in the expanding leaves, with the bulk of the activity found in the tips of both the leaves and the cotyledons. The differences in the root tissues were more pronounced. After 48 h the control exhibited GUS activity throughout the primary root, with increased activity at the points of initiation of the secondary roots and within the elongating secondary roots. Heightened activity was found near the root meristems compared to ANE-treated plants, which exhibited much weaker GUS activity in the primary root with fewer and more weakly staining secondary root structures. The differences persisted in the root tissues 5 days following ANE treatment, with reduced GUS activity most prominent in the expanding root section of both the primary and the lateral roots (data not shown).

Additionally, transgenic WS-0 *Arabidopsis* harboring the CK-sensitive promoter *ARR5*, which controls *GUS* expression, was visualized on ANE-treated or control

plants. Forty-eight hours after transferring the seedlings on media containing ANE, the histochemical GUS activity was present in nearly all of the tissues of both ANE and control-treated seedlings. There was activity throughout the cotyledons, hypocotyls, and roots, with particularly strong activity in the vascular and meristematic tissues. ANE-treated plants exhibited relatively more intense GUS staining 48 h after treatment relative to the controls, particularly in the cotyledon mesophyll cells, the hypocotyls, and the roots (Fig. 3). Five days after treatment the control plants displayed *ARR5*-driven *GUS* activity, primarily in the vascular tissue, with the activity moving away from the aging tissues toward the newly expanding leaves and at the root meristems (both primary and secondary). Relative to the controls, the ANE-treated seedlings had more intense GUS activity within the newly expanding leaves (notably in the mesophyll cells in addition to the vascular tissues), and intense staining near the shoot apical meristems and the newly formed immature leaves. When roots were examined 5 and 7 days after transfer to the ANE treatment, the heightened *ARR5* expression was maintained, with the primary root staining intensely and increased activity maintained around both primary and secondary root tips relative to the controls (Fig. 3b–d).

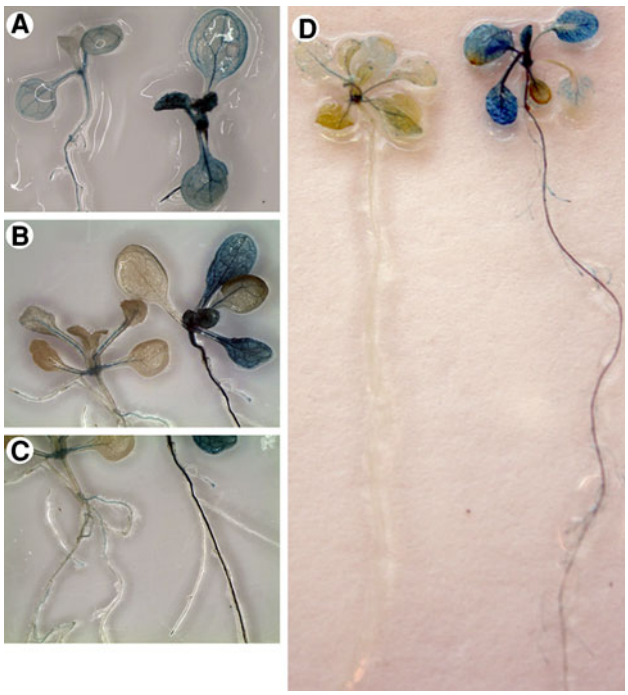

Fig. 3 ANE treatment altered cytokinin distribution in foliar tissues and roots. **a** GUS staining of *ARR5::GUS* transgenic seedlings 48 h after transfer to ½ MS control (*left*) or ANE (0.01 % w/v) medium (*right*). **b, c** Five days after transfer. **d** Intact 12-day-old seedling 7 days after transfer

Effect of ANE on Phytohormone Levels

Since we determined that the levels of phytohormones present in the various commercial seaweed extracts are insufficient to alter plant phenotypes or induce promoter expression in the GUS transgenic *Arabidopsis* plants, we hypothesized that the extracts were altering endogenous, biosynthetic pathways of plants. To this end, we quantified the levels of a complete spectrum of phytohormones in *Arabidopsis* plants grown under highly controlled conditions to minimize biological variations.

Endogenous levels of auxins, including free IAA, indole-3-butyric acid (IBA), and IAA conjugated forms [that is, IAA-aspartic acid (IAA-asp), IAA-glutamic acid (IAA-glu), IAA-leucine, and IAA-alanine], were quantified by UPLC-ESI–MS/MS. Of the auxins detected, only IAA, IAA-asp, and IAA-glu were present in all of the samples, of which IAA was the most dominant auxin representing nearly 90 % of the total detected. There was a high level of variation in the absolute IAA levels between experimental repeats and therefore the data could not be pooled (Supplementary Fig. 3). Despite high levels of variation in auxin levels, similar trends were observed and differences were apparent when treated as ratios of the controls 24 h after treatments (Fig. 4). The overall level of IAA increased in the control plants by approximately 20 % between 24 and 96 h before falling rather sharply by nearly 50 % between 96 and 144 h. The ratios of IAA were reduced in the rosette leaves of ANE-treated *Arabidopsis* by 33 % and 42 % at 24 and 96 h following treatment, respectively ($p < 0.05$), with the ratio falling by 144 h after treatment to a level that was similar to the control-treated plants.

Levels of various CKs, including bioactive forms as well as conjugated ones, were analyzed in the rosette leaves of control and ANE-treated *Arabidopsis* plants. Results show that the bioactive free-base cytokinins, that is, Z (both *cis*- and *trans*-CKs), dhZ, and 2iP, were not detected; however, their precursors ZR and iPA were present in good amounts (except for dhZR which was not detected). Moreover, ZOG (zeatin-O-glucoside), which is another catabolism product of Z, was also found in considerable amounts. Of the CKs detected, the *trans*-CKs were the most abundant at 24 h after ANE treatment, whereas *t*-ZR and iPA were the most abundant, representing nearly 65 % of the total CKs detected. Ninety-six and 144 h after treatment, the *cis*-CKs corresponded to a much greater ratio of the total CK pool, with *c*-ZOG becoming the dominant isomer. Treatment with ANE increased the overall levels of CK in *Arabidopsis* rosette leaves nearly 50 %; moreover, whereas the levels of *cis*-CK isoforms were unaffected, increases of 100, 54, and 76 % were found for *t*-ZR, iPA, and total-*trans* isomers, respectively ($p < 0.05$). Overall, the levels of CK decreased

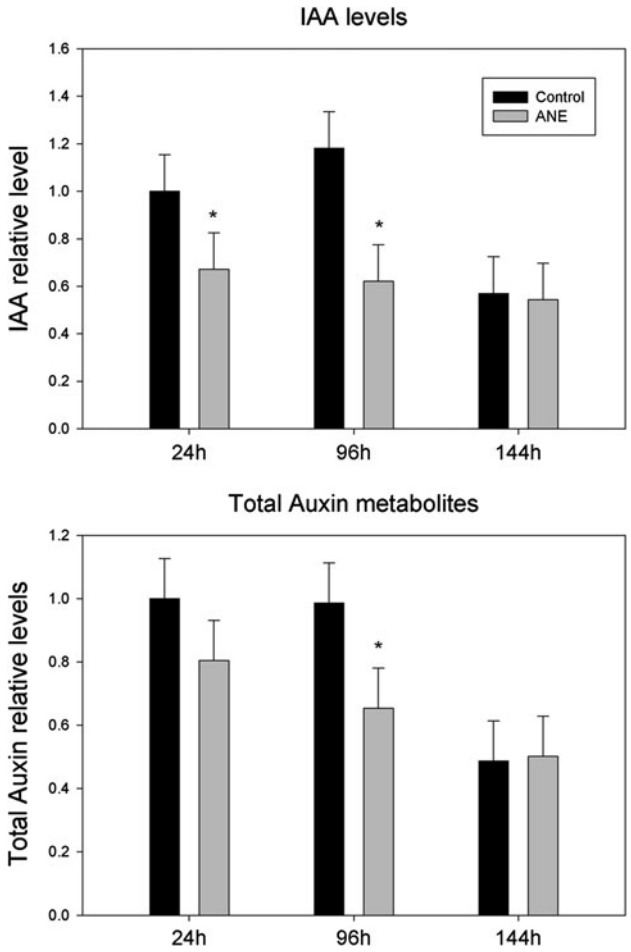

Fig. 4 Relative auxin concentration in rosette leaves of *Arabidopsis* treated with ANE. Endogenous IAA levels relative to the control levels at 24 h (*bar* represents ± SE)

at 96 h in both control and ANE-treated plants, whereas ANE treatments maintained a 40 % increase in total CK relative to 96-h control treatment, corresponding to increases in *trans*-CKs (Fig. 5, $p < 0.05$). Conversely, at 144 h following treatment, there was no significant difference in the total CK levels; however, the levels of *trans*-CKs were slightly higher in the control plants ($p < 0.1$), whereas the *cis*-CKs, in particular, *c*-ZOG, were found to be increased by 40 % in ANE-treated plants ($p < 0.05$). The ANE treatments resulted in an early increase in the overall levels that returned to control levels by 144 h after the treatments.

Because seaweed extracts have been implicated in ABA-mediated responses, we quantified the concentration of ABA and its catabolites in *Arabidopsis* leaves following ANE treatment (Fig. 6). The ABA levels increased with time in both control and ANE-treated plants, increasing by 100 % from 24 to 144 h post treatment, possibly due to age and size of the plants. There was also an increase in the ABA levels in plants that had ANE treatment, showing 40, 23, and 25 % increases at 24, 96, and 144 h, respectively ($p < 0.1$).

Metabolites of ABA were examined to gain insight into ABA homeostasis following ANE treatment. Catabolic products of ABA metabolism through 7′-, 8′-, and 9′-hydroxylation as well as conjugation were examined. Phaseic acid (PA), dihydrophaseic acid (DPA), and ABA glucose-ester (ABA-GE) were the only catabolites detected in the samples analyzed. Similar to ABA, the levels of these catabolites were found to increase with time by more than 100 % from 24 to 144 h. There were relatively large increases of 75 and 86 % at 144 h for PA and DPA in ANE-treated plants, respectively ($p < 0.05$). These increases indicated that the ANE-treated plants were converting the enhanced ABA produced to less active forms to alleviate growth-reducing effects of ABA. The application of ANE increased the ABA-GE levels relative to controls only at 144 h following treatment.

Bioactive gibberellins control a range of functions in plants that include several phenotypes observed in plants treated with seaweeds extracts. A complete suite of bioactive GAs, GA precursor, and GA degradation products was examined in *Arabidopsis* rosette leaves (Supplementary Table 1). Data suggest that representatives of both the non-hydroxylation GA biosynthetic pathway (GA12 → GA15 → GA24 → GA9 → GA4 → GA34) as well as the early 13-hydroxylation pathway (GA53 → GA44 → GA19 → GA20 → GA1 → GA8) are present in small amounts. The presence of larger amounts of GA24 and its further catabolism product GA34 suggests that bioactive forms GA9 and GA4 must have been produced. Smaller amounts of GA53 and GA19 are also present. Results of the analysis show that ANE treatment slightly increased the overall levels of GAs, although not significantly.

Effect of ANE on Gene Expression

Since we observed that ANE treatment altered the endogenous concentrations of auxins, CKs, and ABA in *Arabidopsis*, we proceeded to investigate the effect of ANE on the expression of key genes involved in the metabolism of auxins, CKs, and ABA. In addition, we measured the expression of ABA- and CK-responsive genes to validate findings of phytohormone quantification.

For CK metabolism, we examined the expression of *Arabidopsis IPT* genes involved in the synthesis of bioactive CKs (that is, *t*Z and iP-type CKs) primarily from the methylerythritol phosphate (MEP) pathway, through ATP/ADP IPTs (*IPT3, 4*, and *5*) (Miyawaki and others 2004, 2006), *CK hydroxylase CYP735As* (Takei and others 2004), in addition to the activation genes *CK nucleoside 5′- monophosphate phosphorohydrolase* [*LONELY GUY* (*LOG*) (Kuroha and others 2009)]. The expression levels of *IPT3*, *4*, and *5* were quite low, indicating very low transcript levels in the leaf and

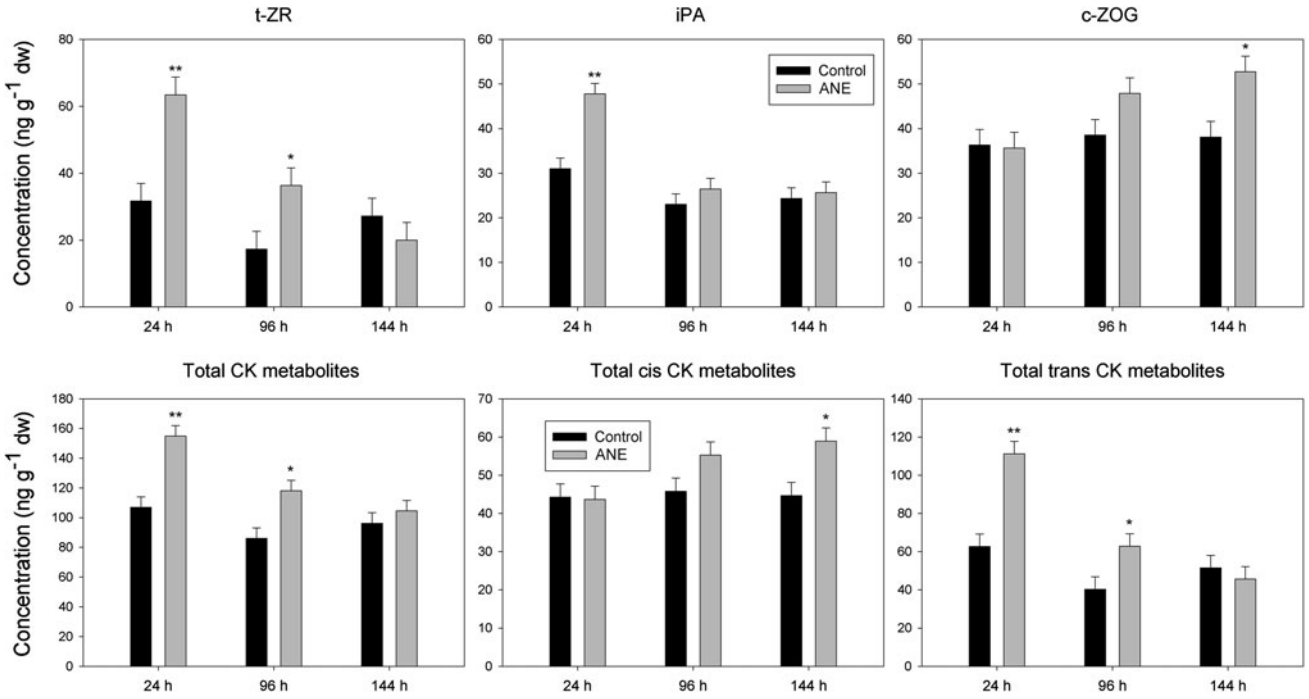

Fig. 5 Effect of ANE treatment on cytokinin concentration in rosette leaves of *Arabidopsis*. Quantification of individual CK metabolites (*top*) and total CK metabolites in addition to total *cis* and *trans* metabolites (*bottom*) from 24, 96, and 144 h following ANE (0.01 % w/v) treatment or control (*bar* represents ± SE)

root tissues examined. However, there were increases in transcript levels of between 1.6- and 3-fold of the control levels following ANE treatment at 24 h which dropped to between 1.4- and 2-fold by 96 h (Fig. 7). Expression of *CK hydroxylases* was subsequently examined; due to the overall low transcript abundance of ATP/ADP *IPTs*, there was extensive variation within our *CYP735A1* measurements, which were not included. However, the expression of *CYP735A2* followed a similar pattern to that of *IPT3*, *4*, and *5* in ANE-treated plants (Fig. 7). Because there was an increase in some *cis*-CKs in ANE treatment at 96 and 144 h, we examined whether their formation was due to isomerization of the existing *trans*-CKs or whether there was delayed activation of the mevalonate (MVA) pathway using tRNA *IPTs*. Transcript profiles of both *IPT2* and *IPT9* were examined, with no significant alteration in expression levels for either gene following ANE application, indicating that the shift towards *cis*-CKs at later time points was not due to MVA pathway activation (Fig. 7).

Final conversion of riboside CK precursors to their highly active free-base CKs is through either a direct conversion through *LOG* genes or a multiple-enzyme process that is not completely understood. Quantification of the transcripts of the most abundant *LOG* genes (*LOG1*, *LOG7*, and *LOG8*) in *Arabidopsis* leaves from control or ANE-treated plants revealed no significant increase in the transcript levels (Supplementary Fig. 4).

Potential increases in total CKs are possible through reduced expression of *CKX* genes. Key genes involved in CK catabolism, *CKX2* and *CKX4*, were examined to determine if CK breakdown was affected due to ANE treatment. Expression of *CKX2* was relatively low and no significant difference was observed between ANE-treated and control plants. The *CKX4* transcript level was more abundant compared to that of *CKX2*, with the expression of both genes increasing with the age of the plant. However, ANE treatment of seedlings reduced the expression of *CKX4* dramatically from 75 % of the control level 24 h following application to less than 25 % of the control levels at 96 and 144 h following application.

Increased *ARR5* expression was observed in response to ANE treatment (Fig. 3). In this experiment we used Wassilewskija (WS-0) backgrounds of *Arabidopsis*, which are impaired in root-based perception of beneficial growth-promoting microorganisms (Ton and others 2001) and may respond differently to the bioactive compounds found in ANE. To determine if similar CK responses were occurring within the Col-0 background, we quantified the *ARR5* transcript following ANE application in *Arabidopsis* Col-0. We observed a greater than twofold increase in *ARR5* transcripts at 24 and 96 h post application before returning to levels similar to that of control at 144 h. In addition, one of the main implications of exogenous CK application or increased endogenous CK levels is the delay of the onset of

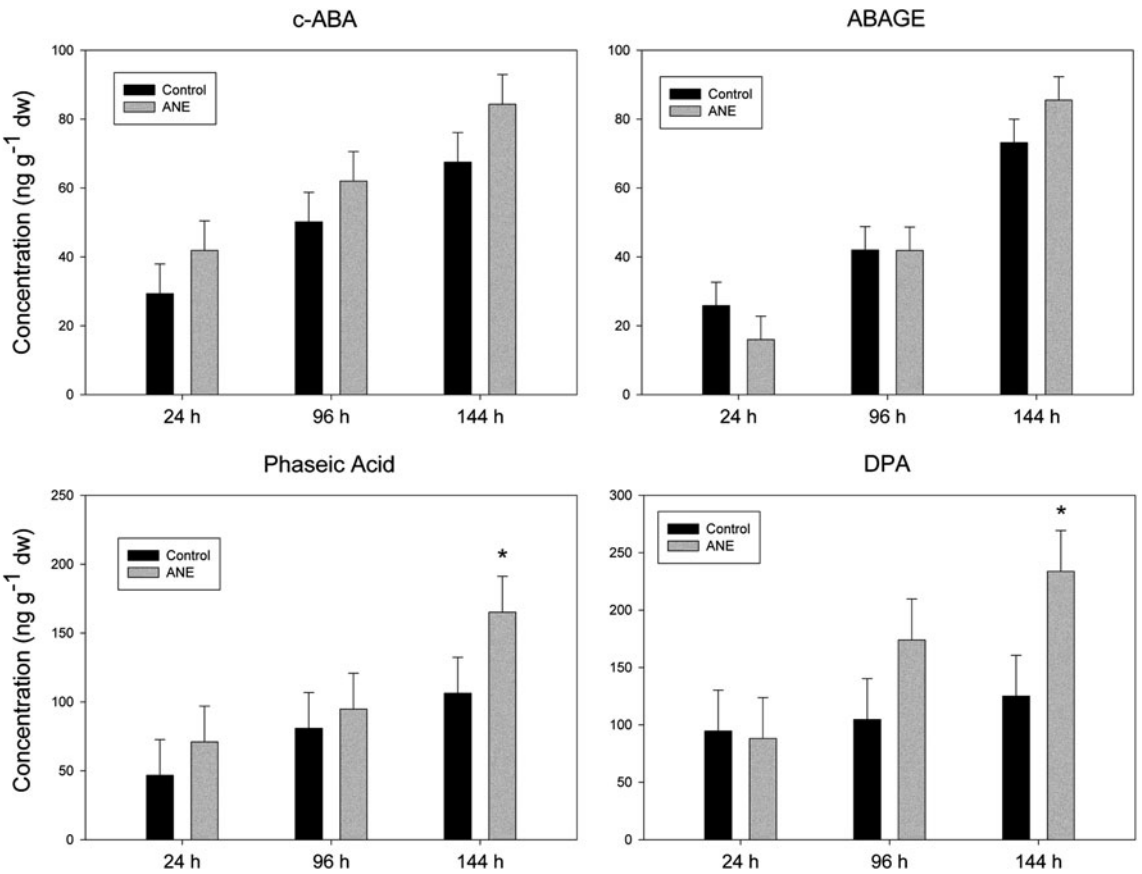

Fig. 6 Effect of ANE treatment on ABA in rosette leaves of *Arabidopsis*. Quantification of ABA and ABA metabolite concentrations in *Arabidopsis* rosette leaves at 24, 96, and 144 h after treatment with ANE (0.01 % w/v) (bar represents ± SE)

senescence. There was a strong inhibition in the transcription of the *Senescence Associate Gene 13* (*SAG13*) at 96 and 144 h following ANE application relative to the control (Fig. 8), indicating a delay in overall leaf senescence due to ANE treatment.

We next examined the effect of ANE on key auxin biosynthetic genes. Transcript levels of the genes *P450 cytochrome oxidases CYP79B2* and *B3*, which are key rate-controlling enzymes for auxin biosynthesis, were examined. In a pattern consistent with the total auxin levels, the expression of *CYP79B2* was repressed by 0.7-fold that of the control at 24 and 96 h before returning back to levels similar to that of control at 144 h (Fig. 9a). However, the expression of *CYP79B3* was nearly identical but there was greater variation between biological replicates with no statistically significant differences beyond 24 h (data not shown). As the maximum suppression of the auxin level was observed 24 h following ANE treatment, the expression of a number of auxin biosynthetic genes was examined, including *indole-3-glycerol phosphate synthase* (*IGPS1*), *phosphoribosylanthranilate transferase 1* (*PAT1*), *tryptophan synthase alpha chain* (*TSA1*), and *nitrilase 1,2* (*NIT1,2*). All of these biosynthetic genes were found to be reduced to below 0.4-fold of control levels except *PAT1*, which was only weakly suppressed by ANE treatment (Fig. 9b).

As a result of a slight increase in the accumulation of the active GA precursor GA24, we examined the expression of the key GA biosynthetic genes *GA3ox1* and *GA2ox2*. The expression of *GA3ox1* and *GA2ox2* was decreased by 40 and 75 %, respectively, in *Arabidopsis* 24 h after application of ANE. The expression of *GA2ox2* returned to control levels at 96 and 144 h, whereas *GA2ox1* transcript abundance was higher than that of the control 96 and 144 h after ANE treatment (Supplementary Fig. 5).

We investigated the expression of key ABA biosynthetic genes to study the mode of action of ANE. These genes included *ABA2* (*xanthoxin dehydrogenase*) and *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*), which regulate the key steps of ABA biosynthesis. Quantification of the transcript levels of ABA biosynthetic genes followed a pattern similar to that of ABA concentration in ANE-treated plants. ANE application resulted in an increased expression of ABA biosynthetic genes. Furthermore, the increase in ABA concentration in ANE-treated plants was confirmed by quantifying the transcript abundance of the

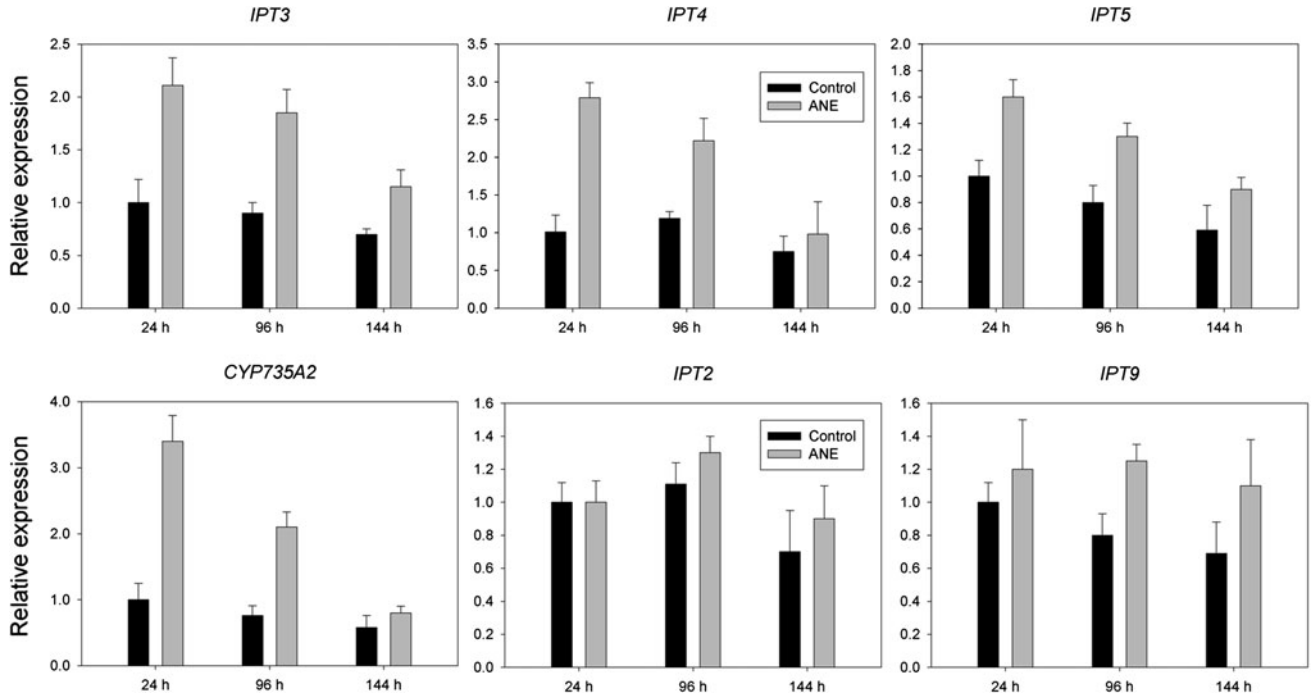

Fig. 7 Expression of the key CK IPT biosynthetic genes acting as part of the methylerythritol phosphate (MEP) pathway (IPT3, 4, and 5) and CK hydroxylase CYP735A2 involved in production of *trans*-CKs in *Arabidopsis* with the tRNA IPTs (IPT2, 9) involved in the formation of *cis*-CKs following ANE application. Data represent the mean ± SE

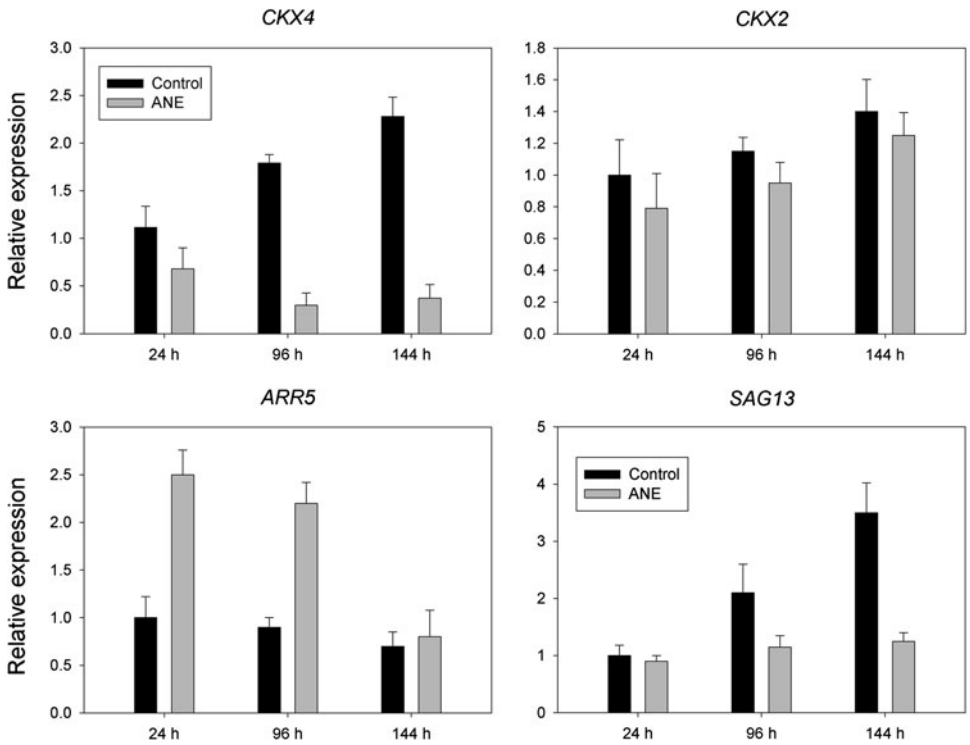

Fig. 8 Expression of the key CK catabolic genes *cytokinin oxidase/hydroxylase CKX4* and *CKX2* (*top*) and expression of the CK-responsive genes *ARR5* and *SAG13* (*bottom*) at the indicated times following ANE application. Data represent the mean ± SE

ABA-responsive gene *RD29a*. There was a significant increase in the transcription of *RD29a* (up to 4-fold) (Fig. 10).

We examined the transcript levels of *CYP707A3*, which encodes a key *ABA 8′-hydroxylase*, to determine if the heightened level of observed ABA catabolic end-products

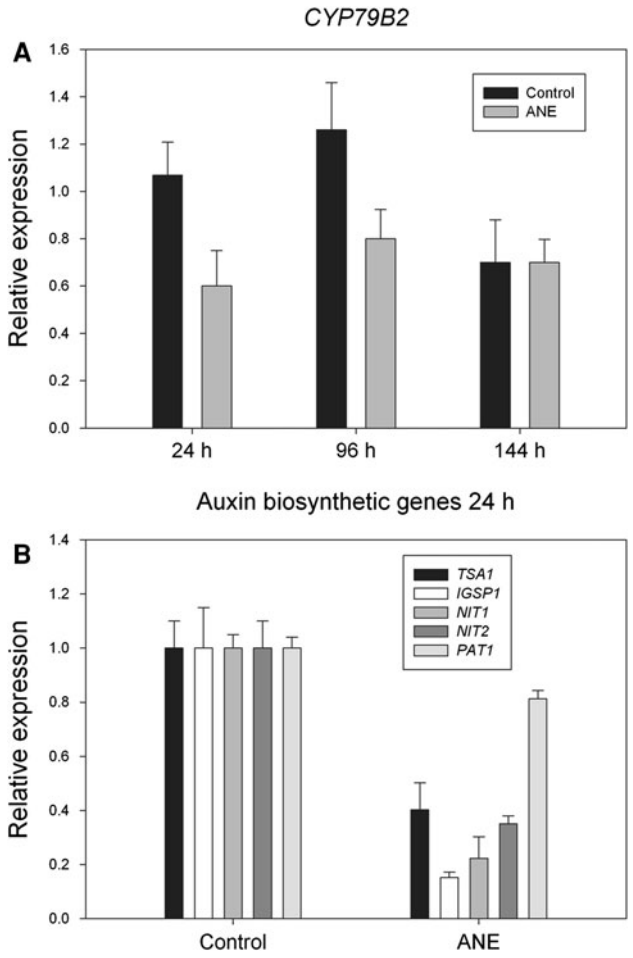

Fig. 9 Auxin biosynthetic gene response to ANE treatment. **a** Expression of the key auxin biosynthetic gene *CYP79B2* following ANE treatment. **b** Expression of auxin biosynthetic genes at 24 h following ANE application. Data represent the mean ± SE

PA and DPA following ANE treatments was due to altered expression of ABA catabolic genes. ANE application initially repressed *CYP707A3* (Fig. 10), consistent with an early increase in ABA, with relatively little change in the levels of PA and DPA (Fig. 6). From 96 to 144 h following ANE treatment, *CYP707A3* transcript levels increased, coinciding with an increase in the ABA catabolite levels at these times (Fig. 6). The strong increase in PA and DPA potentially allows the plants to deal with the growth inhibitory effects of excess ABA under optimal or near-optimal growth conditions.

Discussion

Extracts of *Ascophyllum nodosum* have been used extensively as plant biostimulants in a variety of forms to improve crop performance and yield. Apart from containing naturally occurring plant growth stimulatory compounds such as vitamins, oligosaccharides, and micronutrients, seaweed extracts also have been reported to contain phytohormones (Craigie 2011), with numerous reports indicating that seaweed species (both brown and red seaweeds) contain the same hormones as terrestrial plants (Boyer and Dougherty 1988; Stirk and others 2003, 2004; Yokoya and others 2010). Rayirath and others (2008) and Khan and others (2011) supported this argument, showing that extracts of *A. nodosum*, both whole and organic fractions, induce auxin and cytokinin-like effects using transgenic *Arabidopsis* plants harboring GUS reporters. Here we demonstrated that alteration in plant phenotype following seaweed extract application results from a modulation of biosynthesis, quantity, and ratios of the endogenously produced cytokinins, auxins, and abscisic acid metabolites, rather than from the exogenous phytohormones present within the extracts themselves. There was an overall increase in the concentration of both CK and ABA and their metabolites in *Arabidopsis* leaf tissue and a complimentary decrease in auxin levels following treatment with ANE. This overall increase in endogenous CKs, coupled with a reduction in IAA levels, likely explains previous reports of increased vegetative plant growth and resistance toward drought and salinity stress (Craigie 2011; Khan and others 2009).

Auxins are key hormones involved in promoting elongation of primary roots, initiation of lateral root primordia, and development of formed lateral roots (Aloni and others 2006; Nibau and others 2008; Osmont and others 2007). In addition, ABA antagonizes auxin activity, which can be observed through inhibition of root elongation and lateral root formation, whereas CKs can induce auxin activity in developing tissues or inhibit auxin in established tissues (De Smet and others 2003, 2006; Fukaki and Tasaka 2009; Wasilewska and others 2008). CKs have been shown to inhibit the formation and establishment of an auxin gradient that is required for root elongation and initiation through the reduction in expression of PIN proteins (Ruzicka and others 2009). The inhibitory effects of ABAs on root formation are not fully understood; however, recent evidence suggests that elevated ABA levels inhibit polar auxin transport to the roots under optimal growing conditions, resulting in decreased root formation (Shkolnik-Inbar and Bar-Zvi 2010). Application of ANE resulted in an overall reduction in primary root growth and reduced lateral root formation of *Arabidopsis* plants grown in vitro on a nutrient enriched agar plate system (Fig. 1). This lateral root reduction, typical when CK or ABA are increased, would be further decreased by suppression of auxin biosynthesis and may help to explain the rooting phenotypes observed (Fukaki and Tasaka 2009; Werner and others 2001). Experiments conducted with the ABA- and CK-insensitive mutant *abi4-1* allowed us to conclude that the

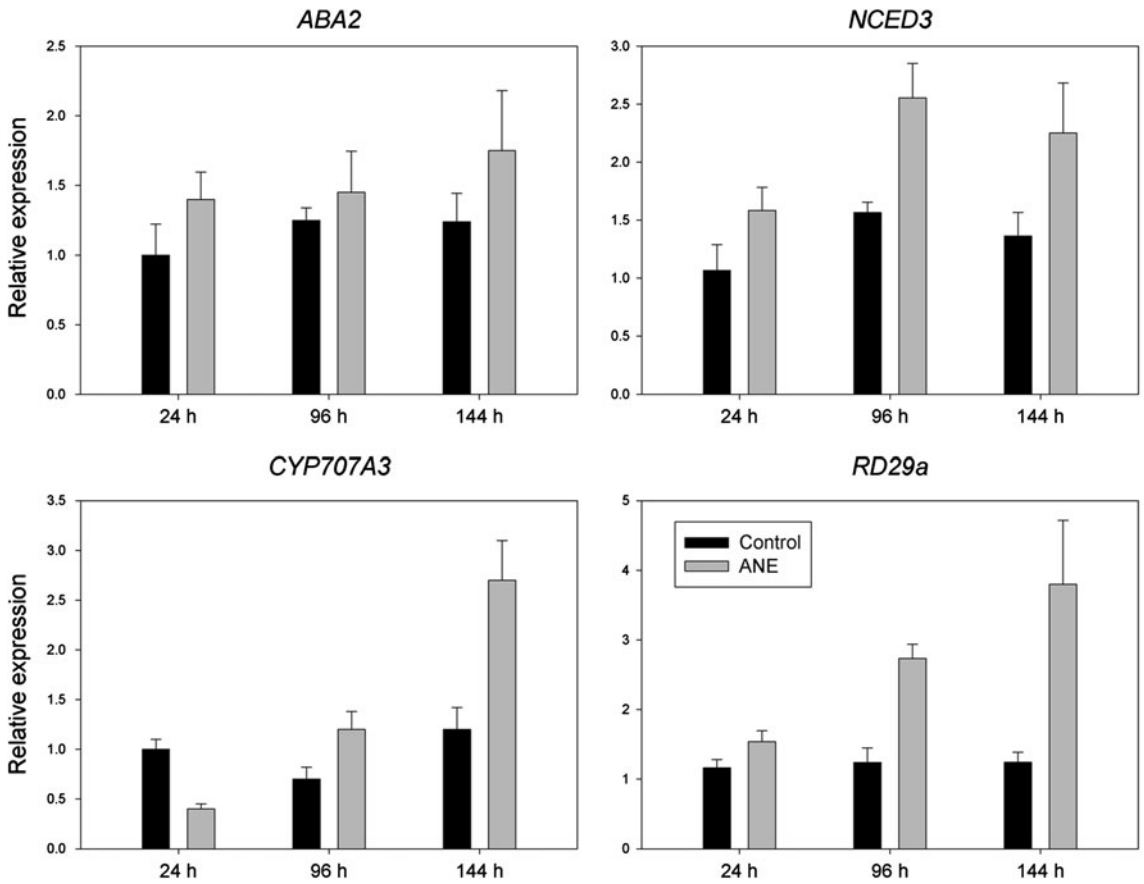

Fig. 10 Expression of the key ABA biosynthetic genes *xanthoxin dehydrogenase* (*ABA2*) and *NCED3*, the ABA catabolic gene *CYP707A2*, and the ABA-responsive gene *RD29a* in *Arabidopsis* leaves at indicated time points following ANE application. Data represent the mean ± SE

inhibition of root branching was due in part to the reduced perception of CK because there was no reduction in primary root length of *abi4-1* plants grown on ANE-containing medium and the reduction in number of lateral roots was similar to the reduction observed with *abi4-1* plants grown on the surface of media containing low concentrations of CKs (Shkolnik-Inbar and Bar-Zvi 2010). To determine if the phytohormone effects were due to levels within the ANE itself, we grew the quadruple mutant *ipt1,3,5,7*, which is deficient in CK biosynthesis but can respond to exogenously applied CKs (Matsumoto-Kitano and others 2008), on media containing ANE and monitored the shoot and root growth. There was less reduction in primary root growth in *ipt1,3,5,7* plants grown on media supplemented with ANE than in the wild-type Col-0 plants, which is consistent with our hypothesis that the observed CK-enhanced phenotypes are not due to hormones coming from the ANE itself.

Previous reports indicated an increase in activation of the synthetic auxin-responsive promoter *DR5* and an increase in root formation in *Arabidopsis* following application of organic subfractions of alkaline ANE (Rayirath and others 2008), whereas the use of the whole aqueous ANE resulted in activation of the CK-responsive promoter *ARR5* (Khan and others 2011). Strong antagonistic relationships exist between CK and auxin biosynthesis (Bishopp and others 2011; Moubayidin and others 2009; Ruzicka and others 2009; Werner and others 2010), indicating that it would be unlikely that supplementing plants with a single beneficial compound would lead to accumulation of both CKs and auxins. As the seaweed extracts contain a variety of different organic components that could potentially stimulate plant growth through a number of distinct pathways, the individual effects found within ANE could be enhanced via removal or augmentation of the different organic components through organic purification and separation to provide distinct bioactive compounds, whereas the whole ANE exhibits a dominant CK response. Consistent with the decreased levels of IAA within the treated tissues, there was also a strong decrease in auxin biosynthetic genes, with many of the key genes being downregulated by as much as 50 % 24 h after application of ANE.

Biologically significant levels of CKs and cytokinin-like components have been reported to be the most dominant plant growth regulators within seaweed extracts produced from different sources (Stirk and van Staden 1996; Stirk

and others 2003, 2004; Yokoya and others 2010). However, when the CK levels were directly quantified by UPLC-ESI–MS/MS, we found the levels of CKs to be biologically insignificant at levels recommended for application (Table 1). These apparent contradictions may be due to the method by which CK levels were detected, results often relying on callus growth studies using known quantities of CKs to generate a standard curve (Stirk and van Staden 1996). Callus growth assessment does not actually measure the levels of CKs present in the samples; rather it measures the inducible "cytokinin-like" activities that could be induced by responses within the plant tissues themselves. Consistent with the findings presented here, the levels of CKs increased strongly in *Arabidopsis* rosettes within 24 h of ANE application. Highly active, free-base CKs exist in relatively low abundance in all tissues examined, presumably due to their high level of activity and detrimental effects at high concentrations. The dominant forms detected were those of the riboside forms tZR and iPA (precursors of the biologically active forms Z and 2iP) at 24 and 96 h following ANE treatment, whereas the catabolite of *cis*-Z, the *cis* O-glucoside c-ZOG, appeared at much higher concentrations after 144 h. This increase was likely due to heightened induction of the MVA CK biosynthetic genes *IPT3*, *4*, and *5* in concert with the down regulation of the *CKX* genes, in particular *CKX4*. Alteration in the total expression levels of *IPTs* or *CKXs* has previously been demonstrated to alter the total accumulation of CKs in a variety of plants (Bartrina and others 2011; Ghanem and others 2011; Guo and others 2010; Merewitz and others 2011; Rivero and others 2010), and therefore likely to explain the metabolic alteration in CK profiles observed in ANE-treated *Arabidopsis*.

We observed an increase in the levels of ABA and ABA catabolic metabolites in rosettes following application of ANE (Fig. 6). The differences in levels between the treatments increased with time, with larger differences observed in the tissues collected at 96 and 144 h post treatment. Increased ABA metabolites coincide with a concurrent increase in ABA biosynthetic genes *ABA2* and *NCED3* (Fig. 10). Since ABA is known as the key phytohormone involved in abiotic stress tolerance (Nambara and Marion-Poll 2005), the increased levels found in ANE-treated plants could potentially explain many of the reports on plants with improved tolerance to freezing, salinity, heat, and drought following treatment with *A. nodosum* extracts (Mancuso and others 2006; Rayirath and others 2009; Spann and Little 2011; Zhang and Ervin 2004, 2008). In contrast to ABA's benefits to plants under stress conditions, ABA is a potent inhibitor of plant growth through regulating stomatal closure and inhibiting root growth (Nambara and Marion-Poll 2005). Although the increase in ABA was higher following ANE treatment, we observed only moderate inhibition of root growth. The lack of growth inhibition may be explained by accumulation of relatively high levels of the catabolic products of ABA degradation following ANE treatment (Fig. 6), mainly PA and DPA, which exhibit relatively low levels of biological activity (Sharkey and Raschke 1980). The accumulation of both PA and DPA is coupled with an increase in the transcript levels of the key *ABA 8′-hydroxylase CYP707A3* that irreversibly deactivates ABA to PA (Okamoto and others 2006, 2011). *CYP707A*s are strongly induced in tissues with excess free ABA present (Kushiro and others 2004) and potentially allow the plants to deal with the growth inhibitory effects of increased ABA under optimal or near-optimal growth conditions, including conditions following treatment with ANE.

We can conclude that root application of ANE resulted in alteration in the endogenous phytohormone biosynthesis and their relative ratios within the plant, which resulted in the beneficial phenotypes that have been reported previously in ANE-treated plants. These findings do not directly contradict previous reports of phytohormones present within the extracts themselves, with many of the previous bioassays for phytohormones examining growth parameters that would also be sensitive to upregulation of the endogenous pathways (Stirk and van Staden 1996; Stirk and others 2003). Further research is required to determine the different bioactive compounds within the ANE that lead to activation of the different biosynthetic pathways, and to determine if the effects are consistent between extracts of different seaweeds and across different plant species.

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