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Toward the molecular understanding of the action mechanism of *Ascophyllum nodosum* extracts on plants

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Abstract

The importance of biostimulants, defined as plant growth-promoting agents that differ notably from fertilizers, is increasing steadily because of their potential contribution to a worldwide strategy for securing food production without burdening the environment. Based on folkloric evidence and ethnographic studies, seaweeds have been useful for diverse human activities through time, including medicine and agriculture. Currently, seaweed extracts, especially those derived from the common brown alga *Ascophyllum nodosum*, represent an interesting category of biostimulants. Although *A. nodosum* extracts (abbreviated ANEs) are readily used because of their capacity to improve plant growth and to mitigate abiotic and biotic stresses, fundamental insights into how these positive responses are accomplished are still fragmentary. Generally, the effects of ANEs on plants have been attributed to their hormonal content, their micronutrient value, and/or the presence of alga-specific polysaccharides, betaines, polyamines, and phenolic compounds that would, alone or in concert, bring about the observed phenotypic effects. However, only a few of these hypotheses have been validated at the molecular level. Transcriptomics and metabolomics are now emerging as tools to dissect the action mechanisms exerted by ANEs. Here, we provide an overview of the available *in planta* molecular data that shed light on the pathways modulated by ANEs that promote plant growth and render plants more resilient to diverse stresses, paving the way toward the elucidation of the *modus operandi* of these extracts.

Keywords Biofortification · Biofertilizer · Crops · *Arabidopsis thaliana*

Introduction

The historically excessive use and misuse of agrochemicals have resulted in environmental pollution, health concerns, and the development of resistant plant pathogens. As a consequence of increased environmental awareness, the application of synthetic agents to guarantee optimal crop yields is now regarded as less favorable and has been translated into a policy adjustment. For instance, the European government has an EU directive to limit nitrate application (91/676/EEC) and a new directive has been established to ban all persistent, bio-accumulative, or toxic pesticides by implementing a more integrated approach by 2020 (2009/128/EC). However, despite the known adverse environmental effects of inorganic fertilizers, the need to increase the efficiency of agricultural practices to meet the world's food demand propels the global application of fertilizers at an anticipated rate of 1.9% per year to reach up to 200 million tonnes by the end of 2020 (FAO 2017). Although organic farming has been proposed as an environmentally friendly alternative for industrial agriculture, the major disadvantage of this low-input production system is that the yields are significantly lower (estimated 5 to 34%) than those of conventional agriculture, mainly due to a high biotic pressure combined with nutrient limitation (Seufert et al. 2012). Thus, any method that can improve plant nutrient

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capture efficiency in a reduced input system and simultaneously ameliorate the resilience against abiotic and biotic stresses will increase the currently defective agro-environmental balance and positively contribute to crop productivity and agricultural sustainability.

One approach that could meet these requirements and that is steadily gaining interest is the implementation of biostimulants. In 2016, the global biostimulants market was valued at 1.79 billion USD and is projected to reach 3.29 billion USD by 2022 at a compound annual growth rate of 10.43% from 2017 to 2022 (https://www.marketsandmarkets.com/Market-Reports/biostimulant-market-1081.html). However, the first report on "biogenic stimulators" that affect metabolic and energetic processes in humans, animals, and plants dates already from 1933 and since then the terminology and the meaning of this concept have evolved (du Jardin 2015; Yakhin et al. 2017). The European Biostimulant Industry Council (EBIC) states that "Plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality." Furthermore, according to EBIC, biostimulants are distinguished from traditional crop inputs based on the following characteristics: (i) they operate through mechanisms that are distinct from those of fertilizers, regardless of the presence of nutrients in the products; (ii) they differ from crop protection products because they act only on the plant's vigor and do not directly act against pests or diseases; and (iii) they complementarily stimulate crop production besides nutrition and protection (http://www.biostimulants.eu). To clearly distinguish biostimulants from the existing legislative product categories, the following definition of a biostimulant has been proposed: "a formulated product of biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds." (Yakhin et al. 2017).

The most commonly used biostimulants include microorganisms, humic acids, fulvic acids, protein hydrolysates, amino acids, and seaweed extracts (Calvo et al. 2014), with seaweed extracts as the fastest growing biostimulant product on the market (Sharma et al. 2014). Seaweeds, well known for their applications in food and medicine (Dillehay et al. 2008), have been utilized for centuries in their unprocessed form as soil conditioners in agricultural settings and their benefits as sources of organic matter and nutrients have been valued for a long time (Craigie 2011). Currently, approximately 28.5 million tonnes of seaweed products are produced annually (FAO 2016), a small portion of which is processed to seaweed formulations applied as plant nutrient supplements and biostimulants. The majority of these seaweeds are commercially harvested in 35 countries, with China, Indonesia, the Philippines, Korea, and Japan as dominant players. In Europe, macroalgae are collected from natural habitats in France, Ireland, Norway, Portugal, and Spain, with small-scale cultivation in France (Sharma et al. 2014; Buschmann et al. 2017). Brown algae (Phaeophyta), including *Fucus* spp., *Laminaria* spp., *Sargassum* spp., *Ecklonia* spp., *Durvillaea* spp., and *Turbinaria* spp., are the most commonly used species for agriculture and for commercial biostimulant production, because they can reach high biomass levels and are widespread (Khan et al. 2009; Craigie 2011; Sharma et al. 2014; Bulgari et al. 2015; Yakhin et al. 2017). Unprocessed seaweeds and their extracts can influence plant growth indirectly by affecting the physical and chemical soil properties, by acting as chelators, and by modifying the soil microbiota, resulting in improved soil texture, water holding capacity and overall soil health (Khan et al. 2009; Craigie 2011; Calvo et al. 2014; De Pascale et al. 2017; Abbott et al. 2018). The direct benefits of seaweed applications on plants are increased germination rate; enhanced root growth; extra shoot biomass; improved nutrient use efficiency; early flowering; delayed senescence; increased chlorophyll, flavonoid, and nutrient contents; improved tolerance to abiotic (drought, salinity and freezing) and biotic (nematodes, fungi, viruses, bacteria and insects) stresses; superior fruit yield; and enhanced post-harvest quality (Khan et al. 2009; Craigie 2011; Quilty and Cattle 2011; Vera et al. 2011; Calvo et al. 2014; Sharma et al. 2014; Battacharyya et al. 2015; Bulgari et al. 2015; De Pascale et al. 2017; Van Oosten et al. 2017; Abbott et al. 2018).

Among the brown algae, *Ascophyllum nodosum* (L.) Le Jolis, also known as rockweed, has attracted a lot of attention and is sustainably harvested along the North Atlantic coastline (Ugarte and Sharp 2012). *Ascophyllum nodosum* extracts (hereafter designated ANEs) are not only implemented in food and biotechnological applications but also used in agricultural practices. Indeed, because most of the commercially available alga-based products are ANEs, they are the best extracts to decipher the action mechanism of plant growth stimulation and stress mitigation. Currently, nearly 47 companies are engaged in producing commercial ANEs for agricultural applications (Van Oosten et al. 2017). Based on a wealth of physiological data gathered over close to 70 years of research, the biostimulant activity on a wide variety of plants and crops, including trees, cereals, fruits, vegetables, and ornamentals, herbaceous and woody species alike (Sharma et al. 2014; Battacharyya et al. 2015; Bulgari et al. 2015; Abbott et al. 2018), has been attributed to the different inherent biochemical characteristics of these algae. Nevertheless, the pathways triggered by the identified bioactive compounds are often unknown and, therefore, synergistic activities are predicted due to their low concentrations. Known bioactive compounds in ANEs include poly- and oligosaccharides absent in plants, including laminaran, fucan, and alginate; betaines; sterols;

vitamins; amino acids; macro- and micronutrients; phytohormones, such as abscisic acid, cytokinins, and auxins; and unidentified compounds with hormone-like activities (Khan et al. 2009; Craigie 2011; Quilty and Cattle 2011; González et al. 2013; Calvo et al. 2014; Sharma et al. 2014; Yakhin et al. 2017). As the ANE composition is determined by location, season, and physiological status at harvest, and the often proprietary production procedure, the diversity of these commercial seaweed extracts is consequently broad. Moreover, application rate, frequency, and timing vary with plant species, geographic location, and environmental conditions (Craigie 2011; Quilty and Cattle 2011; Sharma et al. 2014; Bulgari et al. 2015). Additionally, the information on the composition of commercial ANE biostimulants is based on total solid and ash contents only that are insufficient quality parameters. Therefore, to predict their performance, the level of key components should be provided as well (Goñi et al. 2016). Thus, to stimulate the adoption of ANEs in mainstream agricultural management practices, consistency and magnitude of the ANE responses need to be normalized and it has to be specified which product will meet which specific need. Besides standardization of the extraction procedure (Sharma et al. 2014; Povero et al. 2016), a complementary approach to attain robustness of the biostimulant claim of ANEs is to unravel their action mechanism and to identify markers that can be employed to test their performance.

As the positive growth effects of seaweed extracts or formulated seaweed products on higher plants are seemingly partly independent of their manurial value and of their micronutrient and phytohormone contents, endogenous in planta processes are thought to be altered upon treatment. In the following sections, we will give a chronological, experimentally detailed, and plant-centered overview of the molecular studies that have been published to get insight into the pathways that are involved in ANE-induced plant growth promotion and in resilience against abiotic and biotic stresses. Overall, the methods used to unravel the molecular basis of these effects include the development of fast biosassays based on hormone-responsive promoters or genes fused to a reporter, such as β-glucuronidase (GUS), the expression analysis of marker genes specific for particular pathways, the use of mutants in specific pathways of interest, and genome-wide expression analysis in treated versus untreated plants. Although many of these methods have been established in the model plant *Arabidopsis thaliana*, they have also been implemented to analyze diverse ANE-induced responses in crops, including oilseed rape (*Brassica napus*), soybean (*Glycine max*), spinach (*Spinacia oleracea*), carrot (*Daucus carota*), tomato (*Solanum lycopersicum*), and cucumber (*Cucumis sativus*). We also provide an extensive table giving an overview of differentially expressed genes discussed in the reviewed papers of diverse phenotypic responses upon ANE treatment (Table 1). To conclude, we allude to bottlenecks in the analysis of the molecular action mechanism of ANEs and end with perspectives on future research that could stimulate the development of these renewable biostimulants as efficient and sustainable agricultural products.

Molecular analysis of ANE-induced plant growth promotion in *Arabidopsis thaliana*

One method to assess the plant growth-stimulating performance of ANEs is to validate their activity in fast and reproducible bioassays that can easily be implemented for large screens of commercial products. In three assays on *Arabidopis thaliana*, accession Columbia-0 (Col-0), the plant growth-promoting activity of aqueous and methanolic extracts of two ANEs, designated ANE1 and ANE2 (Acadian Seaplants Ltd., Darmouth, NS, Canada) (Rayorath et al. 2008a) was evaluated by means of (i) measuring the root tip elongation of 5-day-old seedlings grown for 7 days on half-strength Murashige and Skoog (½MS) medium on vertical plates with 10 and 100 mg L^{-1} of the aqueous extracts and 2 g L^{-1} of the MeOH extract; (ii) determining the fresh weight (FW) increase of 7-day-old seedlings grown for 7 days in liquid ½MS cultures with 1 g L^{-1} of MeOH extract; and (iii) recording plant height and leaf number of 2-week-old plants grown on Jiffy-7 peat pellets for 4 weeks under greenhouse conditions and irrigated once per week with 1 g L^{-1} of the aqueous extracts. Additionally, auxin accumulation and signaling were assayed in 7-day-old seedlings of a transgenic Col-0 line that carried a *DR5::GUS* fusion (Ulmasov et al. 1997). The seedlings were grown in liquid ½MS cultures supplemented with 1% (w/v) sucrose and histochemically stained for GUS activity after a 24-h treatment with 2 g L^{-1} of the MeOH extracts.

Both the aqueous and methanolic fractions of ANE1 and ANE2 stimulated in vitro primary root growth at all concentrations tested, but ANE2 performed better. In contrast, exogenous auxin (10 μM and 100 μM indole-3-acetic acid [IAA]) suppressed primary root growth, but induced lateral root formation. The MeOH fractions of ANE2, but especially of ANE1, increased the FW of the plantlets grown in liquid cultures. Similarly, the aqueous extracts of ANE2 stimulated plant height under greenhouse conditions, but those of ANE1 additionally increased the number of leaves. Finally, the MeOH extracts induced *DR5::GUS* expression in the root and hypocotyl tissues, but to a much lower extent than 25 μM IAA, suggesting that auxin signaling is moderately activated by ANE treatments. Based on these data, transgenic plants grown in liquid cultures were concluded to be useful as fast biosensors to assess growth promotion and to ensure uniform bioactivity in seaweed formulations and/or extract fractions (Rayorath et al. 2008a). Furthermore, it was proposed that the development of other transgenic lines to study the gene

Table 1 Overview of differentially expressed genes triggered by ANE treatments in different experiments

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
Hormone metabolism							
Auxin	*Arabidopsis*	Unknown	Auxin-responsive gene (At2g23170)	Down	Auxin response	Downregulated on day 1 after ANE treatment following salinity stress	[18]
	Arabidopsis	Roots and shoots	DR5::GUS	Up	Auxin accumulation		[1]
	Arabidopsis	Roots and shoots	DR5::GUS	Down	Auxin accumulation		[2]
	Arabidopsis	Rosette leaves	P450 cytochrome oxidase (CYP79B2)	Temporarily repressed	Auxin biosynthesis		[2]
	Arabidopsis	Rosette leaves	P450 cytochrome oxidase(CYP79B3)	Temporarily repressed	Auxin biosynthesis		[2]
	Arabidopsis	Rosette leaves	Indole-3-glycerol phosphate synthase (IGPS1)	Down	Auxin biosynthesis		[2]
	Arabidopsis	Rosette leaves	Phosphoribosylanthranilate transferase 1 (PAT1)	Down	Auxin biosynthesis		[2]
	Arabidopsis	Rosette leaves	Tryptophan synthase α chain (TSA1)	Down	Auxin biosynthesis		[2]
	Arabidopsis	Rosette leaves	Nitrilase 1,2 (NIT1,2)	Down	Auxin biosynthesis		[2]
	Arabidopsis	Complete shoots	Small auxin-upregulated RNA59 (SAUR59)	Up	Auxin response	Common in two treatments	[3]
	Arabidopsis	Complete shoots	Small auxin-upregulated RNA1 (SAUR1)	Up	Auxin response	Only in one treatment	[3]
	Arabidopsis	Complete shoots	Small auxin-upregulated RNA33 (SAUR33)	Up	Auxin response	Only in one treatment	[3]
	Arabidopsis	Complete shoots	Small auxin-upregulated RNA50 (SAUR50)	Up	Auxin response	Only in one treatment	[3]
	Arabidopsis	Complete shoots	Small auxin-upregulated RNA71 (SAUR71)	Up	Auxin response	Only in one treatment	[3]
	Arabidopsis	Rosette leaves	At2g21210 (putative auxin-responsive protein)	Up	Auxin response	Plants were subjected to freezing stress	[4]
	Rapeseed	Roots	Auxin response factor 18	Down	Auxin response		[5]
	Rapeseed	Roots	Auxin induced protein like	Up	Auxin response		[5]
	Rapeseed	Shoots	IAA-amino acid hydrolase 6	Up	Auxin deactivation		[5]
Cytokinin	*Arabidopsis*	Roots and shoots	Arabidopsis response regulator 5 (ARR5)	Up	Cytokinin signaling		[1, 2, 6]
	Arabidopsis	Rosette leaves	Senescence-associated gene 13 (SAG13)	Down	Cytokinin signaling		[2]
	Arabidopsis	Rosette leaves	Isopentenyltransferase 3 (IPT3)	Up	Cytokinin biosynthesis		[2]
	Arabidopsis	Rosette leaves	Isopentenyltransferase 4 (IPT4)	Up	Cytokinin biosynthesis		[2]
	Arabidopsis	Rosette leaves	Isopentenyltransferase 5 (IPT5)	Up	Cytokinin biosynthesis		[2]
	Arabidopsis	Rosette leaves	Cytokinin oxidase 4 (CKX4)	Down	Cytokinin catabolism		[2]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
	Rapeseed	Shoots	Cytokinin dehydrogenase 5 precursor	Down	Cytokinin catabolism	Only 5 days after treatment	[5]
	Rapeseed	Shoots	Cytokinin receptor Cre1b	Up	Cytokinin signaling		[5]
Abscicic acid	*Arabidopsis*	Rosette leaves	Xanthonin dehydrogenase (ABA2)	Up	Abscicic acid biosynthesis		[2]
	Arabidopsis	Rosette leaves	Nine-cis-epoxycarotenoid dioxygenase3 (NCED3)	Up	Abscicic acid biosynthesis		[2]
	Arabidopsis	Complete shoots	Nine-cis-epoxycarotenoid dioxygenase3 (NCED3)	Down	Abscicic acid biosynthesis	Plants were subjected to drought stress; up/downregulated compared with mock	[7]
	Arabidopsis	Complete shoots	Nine-cis-epoxycarotenoid dioxygenase4 (NCED4)	Up	Abscicic acid biosynthesis	Only in one treatment	[3]
	Arabidopsis	Roots and shoots	Response to dessication 29a (COR78/RD29a)	Up	Abscicic acid response	Plants were subjected to salt stress	[8]
	Arabidopsis	Complete shoots	Response to dessication 29a (COR78/RD29a)	Up	Abscicic acid response	Plants were subjected to drought stress	[7]
	Arabidopsis	Rosette leaves	Response to dessication 29a (COR78/RD29a)	Up	Abscicic acid response	Induced by cold	[2, 9]
	Arabidopsis	Rosette leaves	ABA 8′-hydroxylase CYP707A3	Temporarily repressed	Abscicic acid catabolism		[2]
	Arabidopsis	Rosette leaves	At1g63840 zinc finger family protein	Up	Abscicic acid response		[4]
	Arabidopsis	Rosette leaves	At5g01540 putative lectin protein kinase	Up	Abscicic acid response		[4]
	Arabidopsis	Rosette leaves	Arabidopsis homeobox protein 12	Up	Abscicic acid response		[4]
	Arabidopsis	Rosette leaves	Receptor-like protein kinase 1	Up	Abscicic acid signaling		[4]
	Rapeseed	Shoots	Nine-cis-epoxycarotenoid dioxygenase (NCED)	Down	Abscicic acid biosynthesis		[5]
	Rapeseed	Roots	ABA 8′-hydroxylase	Up	Abscicic acid catabolism	Only 5 days after treatment	[5]
	Soybean	Shoots	ABA 8′-hydroxylase CYP707A1a	Up	Abscicic acid catabolism	Plants were subjected to drought stress	[8]
	Soybean	Shoots	ABA 8′-hydroxylase CYP707A3b	Up	Abscicic acid catabolism	Plants were subjected to drought stress	[8]
Ethylene	*Arabidopsis*	Complete shoots	Plant defensin gene 1.2	Up	Ethylene response		[3]
	Arabidopsis	Shoots	Plant defensin 1 (PDF1)	Up	Ethylene response	Upregulated in both ANE treated and stronger in ANE treated bacterially challenged plants	[10]
	Arabidopsis	Complete shoots	Ethylene-responsive transcription factor 2 (ERF2) and ERF7	Up	Disease resistance		[3]
		Complete shoots		Up	Ethylene biosynthesis		[3, 5]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
	Arabidopsis, rapeseed		At5g43450 putative 2-oxoglutarate-dependent dioxygenase				
	Arabidopsis, rapeseed	Complete shoots	At2g25450 putative 2-oxoglutarate-dependent dioxygenase	Up	Ethylene biosynthesis		[3, 5]
	Rapeseed	Complete shoots	Ethylene-responsive transcription factor 2 (ERF2)	Up	Ethylene response		[3, 5]
	Rapeseed	Roots	Ethylene-responsive transcription factor 2 (ERF2)	Down	Ethylene response		[5]
Gibberellic acid	*Arabidopsis*	Rosette leaves	Gibberellin-2-oxidase (GA2ox2)	Temporarily repressed	Gibberellin catabolism		[2]
	Arabidopsis	Rosette leaves	Gibberellin-2-oxidase (GA2ox2)	Down	Gibberellin catabolism		[4]
	Arabidopsis	Rosette leaves	Dwarf and delayed flowering 1 (DDF1)	Down	Gibberellin biosynthesis		[4]
	Arabidopsis	Complete shoots	Gibberellin-regulated protein 1 precursor	Up	Gibberellin response		[3]
	Arabidopsis	Rosette leaves	Gibberellin 20 oxidase 1 (GA20OX1)	Down	Gibberellin biosynthesis		[4]
	Rapeseed	Shoots	Gibberellin-2-oxidase	Down	Gibberellin catabolism		[5]
Salicylic acid	*Arabidopsis*	Rosette leaves	Isochorismate synthase 2 (ICS2)	Up	Salicylic acid biosynthesis	Plants were subjected to freezing stress	[4]
	Arabidopsis	Shoots	Isochorismate synthase 1 (ICS1)	Down	Salicylic acid biosynthesis	Downregulated in both ANE treated and ANE treated bacterially challenged plants	[10]
Nutrient metabolism							
Calcium transport	*Arabidopsis*	Complete shoots	Vacuolar cation/proton exchanger CAX3	Up	Calcium transport	May also transport other metals	[3]
Nitrogen metabolism	*Arabidopsis*	Complete shoots	Nitrate transport regulator 1.5 (NTR1.5)	Up	Nitrate transport		[3]
	Rapeseed	Roots	Nitrate transport regulator 1 (BnNTR1.1 and BnNTR1.2)	Up	Nitrate transport	Also role in auxin transport	[5, 11]
	Spinach	Shoots	Cytosolic glutamine synthetase (GS1)	Up	Nitrate assimilation		[12]
Sulfur transport and metabolism	*Arabidopsis*	Complete shoots	Sulfate transporter 1 (SULTR1)	Up	Sulfate transport		[3]
	Arabidopsis	Complete shoots	Sulfate transporter 5 (SULTR5)	Up	Sulfate transport		[3]
	Arabidopsis, rapeseed	Complete shoots	Sulfate transporter 2.2 (AST56)	Up	Sulfate transport		[3]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
	Rapeseed	Roots and shoots	Sulfate transporter BnSULTR1.1 and BnSULTR1.2	Up	Sulfate transport		[5, 11]
	Rapeseed	Roots and shoots	Sulfate transporter BnSULTR4.1 and BnSULTR4.2	Up	Sulfate transport		[5]
	Rapeseed	Shoots	ATP sulfurylase (ASP)	Up	Uptake of S and Se		[5]
	Rapeseed	Roots and shoots	Serine acetyltransferase (SAT)	Up	Synthesis of cystein		[5]
Copper transport	*Arabidopsis*	Complete shoots	Copper transporter 2 (COPT2)	Up	Copper transport		[3]
	Rapeseed	Roots and shoots	Copper transporter 2 (COPT2)	Up	Copper transport		[11]
Fe/Cd/Mn	Rapeseed	Roots and shoots	Metal transporter NRAMP3	Temporarily Up	Fe^{2+} and Zn^{2+} efflux		[11]
Photosynthesis and chloroplast functioning							
Plastid division	Rapeseed	Roots	MinE	Up	Plastid division regulator		[5, 11]
Photosynthesis-related proteins	*Arabidopsis*	Complete shoots	Violoxanthin de-expoxidase1 (VDE1)	Up	Carotenoid biosynthesis	Plants were subjected to drought stress; up/downregulated compared with mock	[7]
	Arabidopsis	Complete shoots	Photosystem II subunit S (PSBS)	Up	Nonphotochemical quenching	Plants were subjected to drought stress	[7]
	Arabidopsis	Complete shoots	Ribulose bisphosphate carboxylase large chain (RBCS1A)	Up	Rubisco	Plants were subjected to drought stress	[7]
	Arabidopsis	Complete shoots	Beta carbonic anhydrase 1 (βCA1)	Up	Regulation of CO_2 diffusion	Plants were subjected to drought stress	[7]
	Arabidopsis	Complete shoots	Aquaporin PIP1;2	Up	Regulation of CO_2 diffusion	Plants were subjected to drought stress	[7]
	Arabidopsis	Complete shoots	Rubisco activase (RCA)	Up	Rubisco	Plants were subjected to drought stress	[7]
	Arabidopsis	Shoots	Chlorophyllase (AtCHL1 and AtCHL2)	Down	Chlorophyll metabolism		[4, 5]
	Rapeseed	Shoots	Protochlorophyllide reductase B	Down	Chloroplast precursor		[5]
	Rapeseed	Roots and shoots	Ferredoxin-2	Up	Chloroplast precursor		[5]
	Rapeseed	Roots and shoots	Carbonic anhydrase	Up	Carbon dioxide transport		[5]
	Rapeseed	Roots and shoots	Ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL)	Up	Rubisco		[5]
Thylakoid	*Arabidopsis*	Shoots	Digalactosyldiacylglycerol synthase 1 (DGD1)	Up	Galactolipid synthesis for thylakoid membranes	Plants were subjected to freezing stress	[4]
Plastoglobule	*Arabidopsis*	Complete shoots	Fibrillin (FIB)	Up	Plastoglobule functioning	Induced by abiotic stress and by ABA	[3]
	Soybean	Shoots	Fibrillin (FIB1a)	Up	Plastoglobule functioning		[8]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
						Induced by abiotic stress and by ABA	
Development							
Cell wall organization	*Arabidopsis*	Complete shoots	Cellulose synthase-like E1 (CSLE1)	Up	Cellulose synthesis		[3]
	Arabidopsis	Unknown	Cellulose synthase-like E1 (CSLE2)	Down	Cellulose synthesis	Downregulated on day 1 after ANE treatment following salinity stress	[18]
	Tobacco BY-2	Suspension cells	Fucosyltransferase	Down	Cell wall organization		[20]
	Arabidopsis	Complete shoots	UDP-glucose 4-epimerase (UGE1)	Up	Carbohydrate metabolism	Also involved in galactose metabolism	[3]
	Arabidopsis	Unknown	Pectin esterases (At1g53840, At3g10720)	Down	Pectin modification	Downregulated on day 5 after ANE treatment following salinity stress	[18]
	Arabidopsis	Unknown	Pectin methylesterase inhibitor (At1g62760)	Down	Pectin modification	Plants were subjected to salinity stress; Downregulated on day 1 after ANE treatment following salinity stress	[13, 18]
	Arabidopsis	Complete shoots	Pectin acetylesterase 8 (PAE8)	Up	Pectin modification		[3]
Cell cycle	*Arabidopsis*	Complete shoots	Phloem protein 2-A11 (PP2-A11)	Up	Carbohydrate metabolism		[3]
Plant development	*Arabidopsis*	Complete shoots	Late embryogenesis abundant 3 (LEA3) and LEA14	Up	Plant development	Also induced under drought stress and ABA treatment	[3]
		Unknown	Late embryogenesis abundant 1 (At5g06760), LEA3 (At1g02820)	Up	Plant development	Upregulated on day 1 after ANE treatment following salinity stress	[18]
		Unknown	Late embryogenesis abundant 1 and 2 group proteins	Up	Plant development	Upregulated on day 5 after ANE treatment following salinity stress	[18]
	Arabidopsis	Complete shoots	No apical meristem (NAM)	Up	Plant development	Also induced under abiotic stress and ABA	[3]
	Arabidopsis	Complete shoots	Tetraspanin-3 (TET3)	Up	Plant development	also induced under cold stress and ABA	[3]
	Rapeseed	Roots	Putative adventitious rooting–related oxygenase	Up	Adventitious rooting	Induced by auxin	[5]
Stress tolerance							
Compatible solute synthesis	*Arabidopsis*	Shoots	Proline dehydrogenase (ProDH)	Down	Proline catabolism	Plants were subjected to freezing stress	[4]
	Arabidopsis	Unknown	Proline dehydrogenase (ProDH, At3g30755)	Down	Proline catabolism	Downregulated on day 5 after ANE treatment following salinity stress	[18]
	Arabidopsis	Shoots		Up	Proline synthesis	Plants were subjected to freezing stress	[4]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
			Delta-1-pyrroline-5-carboxylate synthetase 1 and 2 (P5CS1 and 2)				
	Arabidopsis	Shoots	Starch excess 1 and 4 (SEX1 and 4)	Up	Starch catabolism	Plants were subjected to freezing stress	[4]
	Arabidopsis	Shoots	MUR4	Up	Galactose degradation	Plants were subjected to freezing stress	[4]
	Arabidopsis	Shoots	Galactinol synthase 2 and 3 (GOLS2 and 3)	Up	Carbohydrate synthesis	Plants were subjected to freezing stress	[4]
	Arabidopsis	Shoots	Vacuolar invertase (BETAFRUCT4)	Up	Carbohydrate synthesis	Plants were subjected to freezing stress	[4]
	Cucumber	Shoots	Galactinol synthase (GolS)	Up	Osmoprotectant synthesis		[16]
	Tobacco BY-2	Suspension cells	Betaine aldehyde dehydrogenase (BADH)	Down	Betaine synthesis		[20]
	Tobacco BY-2	Suspension cells	Galactinol synthase 2	Up	Osmoprotectant synthesis	Cells were subjected to freezing stress	[20]
	Tobacco BY-2	Suspension cells	Pyrroline5-carboxylate synthase (P5CS)	Up	Proline biosynthesis	Induced after 2 h of freezing stress, after 6 h in the control	[20]
	Spinach	Shoots	Betaine aldehyde dehydrogenase (BADH)	Up	Betaine synthesis		[12]
	Spinach	Shoots	Choline monooxygenase (CMO)	Up	Betaine synthesis		[12]
Phenolic and flavanoid biosynthesis	Spinach	Shoots	Chalcone isomerase (CHI)	Up	Flavone biosynthesis		[12]
Antioxidant capacity	*Arabidopsis*	Complete shoots	Dihydroflavonol 4-reductase (DRF)	Up	Anthocyan biosynthesis	Plants were subjected to drought stress	[7]
	Arabidopsis	Complete shoots	Superoxide dismutase (SOD)	Up	ROS detoxification	Plants were subjected to drought stress	[7]
	Arabidopsis	Complete shoots	Glutaredoxin-C2 (GRXC2)	Up	Gluthathione-mediated detoxification		[3]
	Arabidopsis	Unknown	Glutathione S-transferase (At5g172200)	Up	Gluthathione-mediated detoxification	Upregulated on day 1 after ANE treatment following salinity stress	[18]
	Rapeseed	Shoots	Glutathione S-transferases	Up	Gluthathione-mediated detoxification	Classes Tau and Phi were upregulated	[5]
	Soybean	Shoots	Glutathione S-transferase (GST)	Up	Gluthathione-mediated detoxification	Plants were subjected to drought stress	[8]
	Tobacco BY-2	Suspension cells	Glutathione S-transferase (GST)	Down	Gluthathione-mediated detoxification		[20]
	Spinach	Shoots	Thylakoid-bound ascorbate peroxidase (tAPX)	Up	ROS detoxification		[12]
	Spinach	Shoots	Monodehydroascorbate reductase (MDHAR)	Up	Ascorbate regeneration		[12]
Stress protein	*Arabidopsis*	Complete shoots	Cold-regulated 47 (COR47)	Up	Stress protection		[3]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
						Induced by cold shock, ABA and drought stress; member of LEA family	
	Arabidopsis	Complete shoots	Cold-regulated 15A (COR15A)	Up	Stress protective protein		[3, 9]
	Arabidopsis	Complete shoots	Dehydration response element B1A (DREB1A)	Up	Transcription factor–induced by abiotic stress	Targets a.o. COR15A	[14]
	Arabidopsis	Unknown	Circadian Clock Associated 1 (CCA1, At2g46830)	Up	Linked to abiotic stress response	Upregulated on day 1 after ANE treatment following salinity stress	[18]
	Arabidopsis	Rosette leaves; complete shoots	Response to dessication 29a (COR78/RD29a)	Up	Stress protective protein	Induced by cold	[2, 9]
	Arabidopsis	Complete shoots	MYB domain protein 60 (MYB60)	Up	Involved in stomata regulation	Plants were subjected to drought stress	[7]
	Arabidopsis	Complete shoots	Responsive to ABA18 (RAB18)	Up	Stress protective protein	Plants were subjected to drought stress; ABA-responsive gene	[7]
	Arabidopsis	Unknown	DRE-binding proteins	Up	Transcription factor induced by abiotic stress	Upregulated on day 1 after ANE treatment following salinity stress	[18]
	Arabidopsis	Roots and shoots	Dehydration response element 2A (DREB2A)	Up	Transcription factor induced by abiotic stress	Plants were subjected to salt stress	[8]
	Arabidopsis	Unknown	Heat Shock Proteins	Down	Induced by exposure to stress	Downregulated on day 1 after ANE treatment following salinity stress	[18]
	Tobacco BY-2	Suspension cells	Activating protein 2	Down	Cold-regulated transcription factor		[20]
	Arabidopsis	Suspension cells	Dehydrins	Up	Stress protein	8 different dehydrins were found to be induced based on protein evidence; tas14 was assessed by qPCR	[19]
	Tobacco BY-2	Shoots	Binding protein (BiP)	Up	Molecular chaperone	Plants were subjected to drought stress	[8]
	Soybean	Shoots	Antiquitin homolog gene (TP55)	Up	Aldehyde dehydrogenase	Plants were subjected to drought stress	[8]
	Soybean	Shoots	Dehydration response element B1A (DREB1B)	Up	Transcription factor–induced by abiotic stress	Plants were subjected to drought stress	[8]
	Soybean	Shoots	BURP domain protein RD22 (RD22)	Up	Dehydration-responsive protein	Plants were subjected to drought stress	[8]
miRNA	*Arabidopsis*	Roots and shoots	miR396a-p	Up	miRNA; targets GR7	Plants were subjected to salt stress	[8]
	Arabidopsis	Roots and shoots	miR169-5p	Down	miRNA; targets NFYA1,2	Plants were subjected to salt stress	[8]
	Arabidopsis	Roots and shoots	miR399a, miR399b, miR399c-3p, miR399c-5p	Down	miRNA; targets UBC24, WAK2	Plants were subjected to salt stress	[8]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
	Arabidopsis	Roots and shoots	miR827	Down	miRNA; targets NLA	Plants were subjected to salt stress	[8]
	Arabidopsis	Roots and shoots	Growth-regulating factor 7 (GRF7)	Down	Regulation of cell expansion; target of miR396a-p	Plants were subjected to salt stress	[8]
	Arabidopsis	Roots and shoots	Nuclear transcription factor Y subunit α1 (NFYA1) and NFYA2	Up	Transcription factor involved in ABA response; target of miR169-5p	Plants were subjected to salt stress	[8]
	Arabidopsis	Roots and shoots	Ubiquitin-conjugating enzyme E2 24 (UBC24)	Up	Phosphate homeostasis	Plants were subjected to salt stress	[8]
	Arabidopsis	Unknown	Wall-associated receptor kinase 1 (WAK1)	Down	Cell surface receptor signaling	Downregulated on day 5 after ANE treatment following salinity stress	[18]
	Arabidopsis	Roots and shoots	Wall-associated receptor kinase 2 (WAK2)	Up	Cell surface receptor signaling	Plants were subjected to salt stress	[8]
	Arabidopsis	Roots and shoots	Nitrogen limitation adaptation (NLA)	Up	Phosphate homeostasis	Plants were subjected to salt stress	[8]
Plant defense	*Arabidopsis*	Complete shoots	Pathogenesis-related 1 (PR1)	Up	Disease resistance		[3]
	Arabidopsis	Shoots	Pathogenesis-related 1 (PR1) and PR5	Down/Up	Disease resistance	Down in ANE-treated plants, up in ANE treated bacterially challenged plants	[10]
	Arabidopsis	Complete shoots	WRKY transcription factors	Up	Salicylic acid response		[3]
	Arabidopsis	Shoots	Plant defensin 1 (PDF1)	Up	Disease resistance	Upregulated in both ANE treated and stronger in ANE treated bacterially challenged plants	[10]
	Arabidopsis	Unknown	Lipid transfer proteins (LTP)	Up	Disease resistance	Upregulated on day 5 after ANE treatment following salinity stress	[18]
	Cucumber	Shoots	Lipoxygenase (LOX)	Up	Disease resistance		[15, 16]
	Cucumber	Shoots	β-1,3-glucanase	Up	Disease resistance		[15]
	Cucumber	Shoots	Peroxidase (PO)	Up	Disease resistance		[15]
	Cucumber	Shoots	Cupi4	Up	Disease resistance		[16]
	Carrot	Shoots	Pathogenesis-related 1 (PR1) and PR5	Up	Disease resistance		[17]
	Carrot	Shoots	Pathogenesis-related 1 (PR1) and PR5	Up	Disease resistance		[17]
	Carrot	Shoots	Nonexpressor of PR genes (NPR1)	Up	Disease resistance		[17]
	Carrot	Shoots	Lipid transfer protein (LTP)	Up	Disease resistance		[17]
	Carrot	Shoots	Chitinase (U52848)	Up	Disease resistance		[17]
	Carrot	Shoots	Chalcone synthase-2 (CHS-2)	Up	Disease resistance		[17]
	Carrot	Shoots	Phenylalanine ammonia-lyase 1 (PAL1)	Up	Disease resistance		[16, 17]

Table 1 (continued)

Category	Species	Tissue	Gene	Up/down	Function	Remark	Reference
	Arabidopsis	Rosette leaves	S-Adenosylmethionine decarboxylase 2 (SAMDC2)	Up	Spermine/spermidine synthesis	Plants were subjected to freezing stress	[4]
	Arabidopsis	Rosette leaves	UDP-glucosyltransferase 73B2 (UGT73B2)	Up	Flavanol conjugation	Plants were subjected to freezing stress	[4]
	Arabidopsis	Rosette leaves	UDP-glucosyl transferase 76C1/2 (UGT76C1/2)	Up	Cytokinin conjugation	Plants were subjected to freezing stress	[4]
	Arabidopsis	Rosette leaves	Salicylic acid glucosyltransferase 1 (SAGT1)	Up	Salicylic acid conjugation	Plants were subjected to freezing stress	[4]
	Arabidopsis	Rosette leaves	UDP-glucosyl transferase 74B1 (UGT74B1)	Up	Glucosinate conjugation	Plants were subjected to freezing stress	[4]

[1] Rayorath et al. (2008a, b); [2] Wally et al. (2013); [3] Goñi et al. (2016); [4] Nair et al. (2012); [5] Jannin et al. (2013); [6] Khan et al. (2011); [7] Santaniello et al. (2017); [8] Shukla et al. (2018); [9] Rayirath et al. (2010); [10] Subramanian et al. (2011); [11] Billard et al. (2014); [12] Fan et al. (2013. [13] Jithesh et al. (2012). [14] Rayirath et al. (2009); [15] Jayaraman et al. (2011); [16] Abkhoo and Sabbagh (2016); [17] Jayaraj et al. (2008); [18] Jithesh et al. (2018); [19] Goñi et al. (2018); [20] Zamani-Babgohari et al. (2019)

expression of marker genes in particular hormone pathways and phenotype-genotype interactions would be valuable.

A transgenic *Arabidopsis* Col-0 reporter line was also used to develop a bioassay for screening cytokinin-like activity in ANEs (Khan et al. 2011) that was faster than the traditional assays, such as the soybean or tobacco (*Nicotiana tabacum*) callus assay (Sanderson and Jameson 1986; Stirk and Van Staden 1997). Seven-day-old *ARABIDOPSIS RESPONSE REGULATOR5 (ARR5)::GUS* seedlings (D'Agostino et al. 2000) were grown in liquid ½MS cultures supplemented with 1, 3, or 5 mL L^{-1} of the commercial alkaline liquid extract Stimplex (Acadian Seaplants Ltd.). Additionally, plants were grown on Jiffy-7 pellets for 3 weeks under greenhouse conditions and sprayed with 1 mL of the 1, 3, and 5 mL L^{-1} Stimplex solution supplemented with 0.02% (v/v) Tween. In both assays, after 48 h of incubation, the plants treated with 3 and 5 mg L^{-1} extract exhibited an increased GUS activity in the root, but especially in the shoot, hinting at the activation of the cytokinin signaling. Although the assay in liquid cultures was more sensitive, the *ARR5* gene was also induced in the greenhouse assay, but less upon the ANE treatment than with 10 μM 6-benzylaminopurine (BAP) used as a positive control. Plant developmental parameters were not measured, hence the observed cytokinin response and the plant growth modulation could not be correlated.

For a long time, the involvement of hormonal pathways in growth improvement of plants upon treatment with seaweed extracts has been postulated. Numerous reports indicate that seaweeds and their extracts contain plant hormones, including abscisic acid (ABA), gibberellins, brassinosteroids, ethylene, auxins, cytokinins, and even strigolactones, leading to the hypothesis that these algal hormones could directly steer growth of terrestrial plants. In support of this assumption, the effectiveness of seaweed extracts depends on the amount applied, with a dose-response curve typical for phytohormones. However, the hormone concentration in the seaweed extracts is low and only relatively small amounts of extracts are applied to plants, questioning this proposition (Stirk and Van Staden 1996; Stirk et al. 2004; Craigie 2011). Although Rayorath et al. (2008a) and Khan et al. (2011) observed an increase in auxin and cytokinin signaling in ANE-treated plants, implying enhanced levels of these hormones, no information on the hormone composition of the ANEs used was provided (Rayorath et al. 2008a; Khan et al. 2011), thus precluding the conclusion that the gene activation is a direct consequence of the hormones in these ANEs.

To take this caveat into account, Wally et al. (2013) combined phytohormone profiling, plant growth bioassays in wild-type and biosynthetic *Arabidopsis* phytohormone mutants, *GUS* expression in transgenic reporter lines, and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis of phytohormone marker genes. All the experiments were done with commercial aqueous alkaline-

extracted ANE powder solution (Acadian Seaplants Ltd.) and the hormone profiling was carried out on 12 commercially available seaweed (*Ascophyllum*, *Ecklonia*, *Macrocystis*, *Durvilea*, and *Sargassum*) extracts. The experiments revealed that the hormone composition and concentration varied significantly. Generally, the hormone levels were very low (pmol g^{-1} dry weight [DW]) and also the range of detected metabolites for the extracts not supplied by Acadian Seaplants Ltd. was limited (maximally 4/16 versus 8/16). It would have been informative to test the effect of these 12 extracts on the root development of *Arabidopsis*, because composition and activity might have been correlated. As the Canadian Atlantic ANEs all contained IAA, ABA, and 2-isopentenyl adenosine (2-iP), the focus was on these three hormone classes. The effect on plant development of the aqueous ANE powder solution was evaluated in a root assay comparable with that of Rayorath et al. (2008a): 4-day-old seedlings were grown on vertical plates on ½MS supplemented or not with 100 mg L^{-1} ANE. The root length was measured after 3, 5, and 7 days of treatment and the lateral root development after 7 days. Besides the analysis of wild-type Col-0 plants, the responses were also assessed of *abi4–1* seedlings that are insensitive to ABA and cytokinin and of the quadruple cytokinin biosynthesis mutant *ipt1,3,5,7*. Additionally, GUS activity was histochemically visualized in 4-day-old seedlings of transgenic *DR5::GUS* and *ARR5::GUS* reporter lines of Col-0 and Wassilewskija-0 (Ws-0), respectively, grown on ½MS and treated with 100 mg L^{-1} ANE for 48 h or 5 days.

The ANE treatment of Col-0 plants inhibited the primary root elongation and reduced the number and length of the lateral roots when compared with control plants, illustrating that the ANE application had a significant impact on the outcome of the treatment (Wally et al. 2013). The mutants, however, responded differently when compared with the wild type: the primary root of *abi4–1* seedlings was insensitive to the ANE treatment, whereas the lateral root initiation, but not the outgrowth, was inhibited. ANE treatment of the *ipt1,3,5,7* mutant still resulted in the inhibition of the primary root elongation, but without obvious effect on the lateral root initiation or elongation. The partial responsiveness of the mutants suggests that some components in the ANE itself cause particular aspects of the root phenotype and indicate that ABA and cytokinins are involved. Curiously, the conclusion has not been drawn that the increased endogenous cytokinin and ABA levels might be responsible for the root phenotype in ANE-treated Col-0. Nevertheless, consistent with the root phenotype in wild-type plants, the GUS activity in the *DR5::GUS* reporter line was reduced in the roots (and shoots) upon the ANE treatment and much fewer lateral root initiation sites were visualized, hinting at an auxin signaling downregulation. Additionally, the ANE treatment strongly and persistently induced the GUS activity in the roots (and shoots) of the *ARR5::GUS* transgenic line, indicating a significant upregulation of the cytokinin response.

As further validation of the hypothesis that endogenous hormone levels are at the basis of the observed root phenotypes, phytohormone profiling and RT-qPCR analysis of selected genes of ANE-treated plants were carried out (Wally et al. 2013). Unfortunately, the experimental setup differed significantly from the previous assays, namely 14-day-old plants treated for 24, 96, and 144 h with ANE were harvested and shoot, instead of root, tissue was analyzed. The phytohormone profiling showed that the IAA levels were lower in the ANE-treated than in the control shoots up to 96 h after the treatment, but were comparable after 144 h. Surprisingly, no cytokinin bases were detected in any of the samples, but zeatin (Z) and 2-iP precursors and total cytokinin content (especially *trans*-zeatin [tZ] and *cis*-zeatin [cZ]) were transiently higher in ANE-treated shoots until 96 h of treatment, whereafter the levels were comparable with those of the controls. Finally, ABA and its catabolites were higher in ANE-treated shoots, but the differences with the control were not significant (except for phaseic acid and dihydrophaseic acid at 144 h). Although these results suggest that the ANE treatment indeed affects endogenous hormone levels, the hypothesis could have been supported when roots had been analyzed, so as to correlate the observed plant responses and the ANE composition (Wally et al. 2013). Additionally, the hormone profiling of the *abi4-1* and the *ipt1,3,5,7* mutants might have provided strong supportive data on the statement that hormone levels in ANEs are too low to cause a direct response in plants.

In line with the histochemical results, the RT-qPCR analysis showed that the *ARR5* expression was upregulated in shoots upon the ANE treatment and that the *SENESCENCE ASSOCIATED GENE13* (*SAG13*) expression was downregulated, both in support of an ANE-enhanced cytokinin response. Although an up to threefold induction was recorded for the cytokinin biosynthesis ATP/ADP *ISOPENTENYLTRANSFERASE3* (*IPT3*), *IPT4*, and *IPT5* genes in response to the ANE treatment, their expression was considered too low to account for the cytokinin signaling activation. Additionally, neither the expression of the tRNA *IPT2* and *IPT9* genes nor of the cytokinin-activating *LONELY GUY1* (*LOG1*), *LOG7*, and *LOG8* genes were significantly altered upon the ANE treatment, further implying that cytokinin biosynthesis in the shoots might probably not play a role in the enhanced cytokinin response. In contrast, the expression of the *CYTOKININ OXIDASE4* (*CKX4*) gene, encoding cytokinin degradation, was strongly repressed after ANE treatment, indicating that the modulation of the cytokinin homeostasis might be part of the action mechanism of this particular ANE. Indeed, the induction of cytokinin biosynthesis genes and the simultaneous downregulation of cytokinin degradation genes are inconsistent with cytokinin addition (Motte

et al. 2013). Wally et al. (2013) concluded that the very modest upregulation of *IPT* gene expression could explain the root phenotype, but they did not consider the possibility that the ANE contained compounds that target *CKX* expression directly lead to the modest accumulation of the endogenous cytokinin levels.

In addition to the analysis of the expression of marker genes of the cytokinin response, the expression of genes implicated in ABA and auxin metabolism was measured as well. The moderate accumulation of ABA and ABA catabolites in the shoot was supported by the ANE-induced expression of the ABA biosynthesis genes, *ABA2* and especially *9-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*), the ABA-responsive gene *RD29a*, and the ABA degradation gene *CYP707A3*, but could not explain the inhibition of the primary root growth and lateral root formation, because the data were obtained for shoots only and not for roots. For the RT-qPCR analysis of auxin-related genes, the *TRYPTOPHAN AMINOTRANSFERASE1* and *YUCCA* genes were not selected (Wally et al. 2013) that represent the indole-3-pyruvic acid route, the main IAA biosynthesis pathway in *Arabidopsis* (Malka and Cheng 2017), but instead the *INDOLE-3-GLYCEROL PHOSPHATE SYNTHASE1*, *PHOSPHORIBOSYLANTHRANILATE TRANSFERASE1*, and *TRYPTOPHAN SYNTHASE* $\alpha 1$ were chosen that are involved in tryptophan synthesis, an IAA precursor, and *CYP79B2*, *CYP79B3*, *NITRILASE1* (*NIT1*), and *NIT2* implicated in the indole-3-acetaldoxime pathway (Malka and Cheng 2017). The expression of all seven selected genes was repressed in the shoot upon ANE treatments. The reduced *DR5::GUS* expression and the decreased IAA level in the shoot suggest downregulation of the auxin pathway. Although the RT-qPCR data concur, they are inadequate to draw conclusions, because the key genes have not been analyzed. Additionally, given that IAA-amino acid conjugates were abundantly present in the used ANE extracts, it would have been interesting to test the expression of the IAA-amidohydrolase genes that hydrolyze the storage forms into the active auxin (Ludwig-Müller 2011).

Altogether, in our opinion, the data presented by Wally et al. (2013) are not sufficient to infer that the hormone effects can be attributed entirely to endogenous hormone modulations in the plant. Furthermore, the opposing effects on root development recorded in the studies of Rayorath et al. (2008a) and Wally et al. (2013) are difficult to interpret, because the plant developmental status varied strongly, hinting at different growth conditions (for instance, the root length of 7-day-old control plants was 15 mm vs 60 mm). Furthermore, only the MeOH fraction and not the aqueous extract of the ANE was tested on the *DR5::GUS* activity (Rayorath et al. 2008a). Additionally, the composition of the ANEs used was not specified, therefore, the difference in ANEs utilized may account for the discrepancy in the results.

A genome-wide expression analysis with GeneChip ATH1 Affimetrix microarrays was carried out on shoot material of *Arabidopsis* Col-0 plants treated for 7 days with two different extracts of a commercial liquid ANE, namely ANE A and ANE B, a neutral and alkaline aqueous extract, respectively (Goñi et al. 2016). The Col-0 plants were grown axenically on MS for 14 days, whereafter they were transplanted to pots with compost/vermiculite/perlite (5:1:1). One day later, the plants were treated with the ANEs with a foliar spray (0.2% *v/v*) and after 7 days the plant height was measured and the leaf number was counted. Samples were taken for microarray analysis. The chemical composition analysis of ANE A and ANE B revealed that the pH change during the extraction process significantly affected the concentration of all key components of the ANEs, including polyphenols, fucoidan, uronics (alginate), laminarin, and mannitol. The largest difference was the 4-fold higher polyphenol level in ANE B than in ANE A, but no information on the hormone levels was provided. Despite these differences in composition, the morphological changes induced with both ANEs consisted of early flowering, longer floral stalks (increased plant height), and increased leaf number, with a stronger response for ANE B than for ANE A. Surprisingly, the genome-wide transcriptional analysis with ANE A revealed that 1011 genes (4.47% of the microarray) were differentially expressed with 599 upregulated, 412 downregulated, and 849 unique to ANE A, whereas only 196 differential genes (0.87% of the microarray) were recorded with ANE B, of which 127 upregulated, 69 downregulated, and 34 unique to ANE B (2-fold change cutoff). Both treatments had 168 differentially expressed genes in common, potentially representing pathways implicated in the shared phenotypical responses. Although no comprehensive list of these genes was made available, based on the MapMan ontology, 32 genes were involved in metabolism (amino acid metabolism, transport, lipid metabolism, and secondary metabolism), 35 gene in development (cell wall, development, and photosynthesis), 14 genes in stress (redox and stress), 5 genes in hormone metabolism, and 82 genes in other pathways (miscellaneous enzymes, protein, and RNA) (Goñi et al. 2016). Metabolism-associated genes upregulated by ANE A and ANA B included transport of amino acids, calcium (*CAX3*), ammonium, and copper (*COPT2*), whereas upregulated development-associated genes comprised cell wall organization (*ATCSLE1*, *UGE1*, and *PAE8*), cell cycle/organization (*AtPP2-A11*, and *FIB*), and plant development (*LEA14*, *LEA3*, *NAM*, and *TET3*). Although no effect was seen on the expression of auxin biosynthesis genes, the expression of *SMALL AUXIN UPREGULATED RNA59* (*SAUR59)*, known to be responsive to auxin, was upregulated by both ANEs, implying a role for auxin signaling in the shoot response in agreement with the results of Rayorath et al. (2008a). No data were presented on the differential expression of cytokinin-related genes.

Finally, Goñi et al. (2016) looked for correlations between the ANE composition and gene expression, but direct relationships between the observed expression patterns and particular component in the ANEs were difficult to find. The upregulation of the cold-induced gene *COR47* was attributed to mannitol present in the ANEs, but could just as well have been caused by, for example, a high sodium concentration in these particular ANEs. They concluded that "the ANE composition −biostimulant activity relationship is complex, and progress in unraveling this relationship will require more comprehensive experiments assessing the effect of the major and minor components of ANE biostimulants singly and in combination." (Goñi et al. 2016).

Molecular analysis of ANE-induced plant growth promotion in crop plants

The effect of ANEs on germination and seedling vigor was tested in several greenhouse bioassays on barley (*Hordeum vulgare*) 'AC Sterling' and 'Himalaya' and a derived gibberellic acid (GA_3)-responsive dwarf mutant *grd2* (Rayorath et al. 2008b). Four fractions were prepared from an alkaline ANE (Acadian Seaplants Ltd): an aqueous solution, a MeOH fraction that was further subfractioned first with chloroform and then with ethyl acetate (EA); all fractions were tested at three concentrations (100 mg L^{-1}, 500 mg L^{-1}, and 1 g L^{-1}). For the evaluation of the ANE effect on seed emergence, seeds were placed in sterile vermiculite and irrigated with the different concentrations of the four fractions. Emergence was recorded every 12 h for 96 h after the first seed emerged. Additionally, seedling growth was measured by determining the shoot and root lengths and their DW 14 days after the ANE treatment. Finally, the impact of ANEs on the mobilization of food reserves from the endosperm to support embryo growth and differentiation was estimated by means of a starch zone-clearing assay for the α-amylase activity. In this process, the α-amylase activity is stimulated by GA_3 and repressed by ABA, thus allowing, by inclusion of the *grd2* mutant, the assessment of whether the ANE fractions contain compounds with GA-like effects.

Both the aqueous and the organic subfractions of the ANE strongly stimulated seed emergence, shoot length, and shoot DW at 1 g L^{-1}, which are very important for seedling establishment, growth, and development in barley, but they did not affect the root development (Rayorath et al. 2008b). To assess which type of component present in the ANE was responsible for these activities, the α-amylase assay was performed under different conditions. For both the wild-type barley and the *grd2* mutant, the organic fractions, but especially the aqueous solution, induced the α-amylase activity, suggesting that the active component in the ANE had GA-like functions. In support to this finding, this capacity was lost or strongly reduced in the organic fractions when activated charcoal was added or when the ANEs were autoclaved, respectively, hinting at an organic and thermolabile nature of the active component. When the α-amylase activity was stimulated by exogenous GA_3, this effect could be completely nullified upon addition of ABA. However, the combination of ANEs with ABA still induced the α-amylase activity, albeit to a lesser extent than ANE alone. As liquid chromatography-tandem mass spectroscopy did not reveal any detectable GA_3 in the ANEs, these data indicate that the ANEs contained compounds with GA-like effects that acted in a GA-independent manner.

The effect of a soluble alkaline ANE powder (Acadian Seaplants Ltd) on the yield and nutritional quality of spinach (*Spinacia oleracea*) 'Unipack 12' was studied by assaying diverse parameters of 6-day-old seedlings grown on ½MS supplemented with 1% (*w*/*v*) sucrose and treated with 100 or 500 mg L^{-1} ANE (Fan et al. 2013). After 21 days of treatment, the effect on growth was assessed by measuring FW, DW, dry matter content (DMC), total soluble protein, and leaf pigments. Total antioxidant capacity, total phenolics, total flavonoids, and phenylalanine ammonia-lyase (PAL) and chalcone isomerase (CHI) activities were scored as well in the spinach shoots. Finally, leaf tissues of 30-day-old plants were analyzed by semi-qRT-PCR to evaluate the expression of genes involved in antioxidant activities.

Treatment with the lowest ANE concentration only resulted in an increase in the plant biomass (FW, DW, and DMC), in the contents of total protein, chlorophyll, phenolics, and flavonoids, and in the antioxidant activity. Whereas the PAL activity was not affected by the ANE treatment, the CHI activity was induced. Of the antioxidant genes tested, the expression remained unchanged of the sucrose phosphate synthase (*SPS*), plastid glutamine synthetase (*GS2*), dehydroascorbate reductase (*DHAR*), and stromal ascorbate peroxidase (*sAPX*) genes. The increased biomass and total protein content might be associated with the enhanced cytosolic *GS1* expression involved in nitrogen assimilation. In contrast, the augmented chlorophyll content could be correlated with an increase in betaine content in the plants caused by the ANE-induced expression of betaine aldehyde dehydrogenase (*BADH*) and choline monooxygenase (*CMO*) that could act additively to the betaine and cytokinin-like ANE components. Finally, the increased total phenolic and flavonoid contents and enhanced antioxidant capacity might be related to the high CHI activity and upregulated expression of glutathione reductase (*GR*), thylakoid-bound APX (*tAPX*), and monodehydroascorbate reductase (*MDHAR*). Thus, the ANE application at the early growth stage of spinach induces particular physiological responses, probably through the phenylpropanoid and flavonoid pathways that not only stimulate plant growth, but also contribute to an enhanced nutritional quality (Fan et al. 2013).

The molecular mechanism of the growth-stimulating effect of AZAL5, an aqueous solution prepared by microrupture

under acidic conditions from freshly harvested *A. nodosum* plants was studied by means of a transcriptomics approach on rapeseed (*Brassica napus*) 'Capitol', grown hydroponically under greenhouse conditions (Jannin et al. 2013). One-week-old plants were treated with 67 mg L^{-1} AZAL5 (day 0) for 30 days (refreshed every 2 days) and different parameters of root and shoot tissues were measured after 1, 3, and 30 days. The determination of the elementary and hormone composition of AZAL5 revealed that C, H, and O were the main elements, but Ca, K, Mg, Na, and S, but not N, were present as well. Although the contribution of AZAL5 to the mineral supply of the hydroponics solution was negligible, 30 days of AZAL5 treatment resulted in a significant increase in the total DW that was attributed more to the root DW than to that of the shoot.

A transcriptomics analysis on a rapeseed Gene Expression Microarray 4 × 44 K containing 31,561 genes, of which 60% unidentified (5-fold change cutoff), did not uncover differential gene expression after 1 day of treatment. After 3 days of treatment, 724 genes were differentially expressed in the shoot and 298 genes in the root, and after 30 days of treatment, 612 differential genes were recorded in the shoot and 439 genes in the root. Classification of the differential genes with the DFCI Gene index annotation tool (http://compbio.dfci.harvard.edu/tgi/tgipage.html) revealed that almost the entire plant metabolism was modified by the AZAL5 treatment. General cell metabolism, carbon metabolism and photosynthesis, stress responses, and nitrogen and sulfur metabolism were affected, but a few differential genes were allocated to metabolic pathways involved in fatty acids, phytohormones, senescence, plant development, and ion transport. To correlate the differential gene expression with physiological changes in the AZAL5-treated plants leading to growth stimulation, mineral and ion analyses were done (total N, total S, nitrate, and sulfate) and nitrate reductase activity, chlorophyll concentration and net photosynthetic rate were determined. After 30 days of AZAL5 treatment, the N content was significantly increased in shoots and roots, coinciding with an enhanced nitrate uptake, resulting from the improved growth (same DW increase rate) and an upregulation of *BnNTR1.1* and especially *BnNTR2.1* expression. Nitrate reductase activity was also induced by AZAL5, but only in the shoots. Similarly, the S content and sulfate level significantly increased in shoots and roots, but the increase rates were much higher than those of DW, hinting at the AZAL5-induced activation of S uptake. In agreement with these findings, the sulfate transporter genes *BnSULTR1.1*, *BnSULTR1.2*, *BnSULTR4.1*, *BnSULTR4.2*, and a serine acetyltransferase were all higher expressed in both tissues. Additionally, genes encoding an ATP sulfurylase and glutathione *S*-transferases of the Tau and Phi classes were only upregulated in the shoot. AZAL5 increased the chlorophyll content, but not the net photosynthetic rate, in line with the downregulation of genes involved in photosynthetic pathways in the shoot. Fluorescence confocal and transmission electron microscopic analyses revealed that the AZAL5 treatment augmented the number of chloroplasts and starch granules, the latter of which had an increased size, implying that the dark photosynthesis reactions leading to C assimilation and starch synthesis were enhanced. This conclusion was supported by the increased expression of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and carbonic anhydrase. Together all the data indicate that in response to the AZAL5 treatment, the N uptake by the rapeseed roots is assimilated directly for growth and is not stored. In contrast, the AZAL5-stimulated S uptake exceeded the growth demand, resulting not only in assimilation, but also in sulfate accumulation (Jannin et al. 2013).

Finally, although Jannin et al. (2013) focused on photosynthesis and nitrogen/sulfur metabolism, they also carried out a hormone profiling of the AZAL5 solution and the AZAL5-treated plants. In the AZAL5 solution, low levels of IAA, ABA, and 2-iP were detected. The IAA level was 20-fold lower than that of the Canadian ANEs, whereas the amounts of ABA, 2-iP, and isopentenyl adenosine (iPR) were threefold higher, threefold lower, and 30-fold lower (Wally et al. 2013). Hormone profiling of the shoots and roots, at 1, 3, and 30 days after AZAL5 treatment revealed that the IAA and ABA contents did not differ from the controls. These findings are in agreement with the downregulation of a putative auxin response factor gene and a putative 9-*cis*-epoxycarotenoid dioxygenase and neoxanthin cleavage enzyme-like protein gene implicated in ABA biosynthesis in shoots. However, the unaltered auxin levels in the roots were not consistent with the large increase in the root biomass and the upregulation of a putative adventitious rooting-related oxygenase and an auxin-induced protein in root tissues, hinting at an increased auxin response. In contrast to the data obtained for *Arabidopsis* (Wally et al. 2013), the bioactive cytokinin bases Z and 2-iP were found in rapeseed shoot tissues, but the concentration of their biosynthetic precursors occurred at over 100-fold lower concentrations (Jannin et al. 2013). The Z content in AZAL5-treated shoots was higher only after 3 days, whereas the *trans*-zeatin riboside (tZR) content was lower for all three time points measured, and the amounts of *cis*-ZR (cZR), 2-iP, and iPR did not differ. Interestingly, at the gene expression level, the cytokinin perception seemed to be upregulated, whereas a putative cytokinin degradation *CKX* gene and several senescence-associated proteins were downregulated, implying an enhanced cytokinin response in the shoot, consistent with the increased chlorophyll content and chloroplast number and the improved growth. In roots, the Z levels were comparable with those of control plants, but the ZR and cZR levels were lower upon the AZAL5 treatment. The iPR level was higher after 1 day of AZAL5 treatment, remained unchanged after 3 days, and was lower after 30 days than that of the control. However, the 2-iP levels were extremely enhanced upon the

AZAL5 treatment at all three time points (up to almost 600% after 3 days of treatment), but none of the differentially expressed genes pointed toward an increased cytokinin response, in agreement with the root system expansion upon the AZAL5 treatment.

As a continuation, the impact of AZAL5 on biofortification in hydroponically grown rapeseed was analyzed in the same experimental setup by measuring the level of micronutrients in root and shoot tissues, especially Cu, Fe, Mg, and Zn, combined with a microarray transcriptomics and RT-qPCR analysis (Billard et al. 2014). Accordingly, after 30 days of treatment with AZAL5, the significantly increased total DW was attributed mainly to the root and less to the shoot DW and the number of chloroplasts was higher in the treated than in the untreated shoots. Nutrient analysis revealed that the relative amounts of macronutrients (N, K, S, P, and Mg) and micronutrients (Fe, Na, Mn, B, Si, Zn, and Cu) increased upon AZAL5 treatment. The uptake of Si, P, and N (designated group II elements) was in accordance with the growth rate, whereas that of Na, Mn, Cu, and S (group I nutrients) exceeded that required for growth, thus leading to accumulation. The concentrations of K, Fe, and Zn (group III elements) did not change for the whole plant, but differed for shoots and roots, suggesting that the AZAL5 treatment affected the root-to-shoot translocation within the plants. In contrast, the microarray analysis revealed 200 differentially expressed genes 1 day after the AZAL5 treatment, increasing to 1630 genes after 3 days and 1717 genes after 30 days, hinting at a stronger impact in this experiment (Billard et al. 2014). In agreement with the increased N and S contents in AZAL5-treated plants, the transporter genes *BnNRT1.1*, *BnNRT2.1*, *BnSULTR1.1* and *BnSULTR1.2* were upregulated. Similarly, the expression of the Cu^{2+} transporter gene *COPT2* was upregulated both in root and shoot tissues, especially after 3 days of AZAL5 treatment, corresponding with the increased Cu concentrations. Furthermore, the downregulation of *COPT2* after 30 days of treatment illustrates the establishment of a negative feedback to prevent Cu^{2+} toxicity. The expression of the Fe^{2+} transporter gene *IRT1* did not change in response to AZAL5 in roots or shoots at any time point, supporting a steady-state root uptake. Despite the increased Mg^{2+} concentrations, no differential expression was recorded for the Mg^{2+} transporter gene *MRS-10*. The transient upregulation of the less specific efflux transporter *NRAMP3* in roots only after 1 day of AZAL5 treatment might be the reason for the enhanced root-to-shoot translocation of Fe^{2+} and possibly Zn^{2+}. As the hormone and nutrient levels in AZAL5 were too low to account for the observed effects on rapeseed, Billard et al. (2014) concluded that macromolecules, such as the polysaccharides laminaran or fucoidan, or a synergistic activity of various compounds might trigger the responses. In that context, it is interesting to note that the transcriptional modification induced by the humic acid extract HA7 was 50% in common with that triggered by AZAL5 in rapeseed (Billard et al. 2014), whereas only a 13% overlap in the differentially expressed genes was found when the effect of two ANEs was compared on the *Arabidopsis* gene expression (Goñi et al. 2016).

Molecular analysis of ANE-induced freezing stress tolerance in *Arabidopsis* and tobacco

Although the positive impact of the application of seaweed extracts to alleviate the detrimental effects associated with diverse abiotic stresses has been documented for a long time in many plant species (Van Oosten et al. 2017), only a few studies aimed to unravel the molecular mechanisms underlying this effect. To get insight into the ANE-induced freezing tolerance, Rayirath et al. (2009) and later Nair et al. (2012) used an in vivo peat pellet-freezing assay with *Arabidopsis* Col-0 plants. An aqueous solution and diverse organic fractions (MeOH fraction and sequential subfractions with hexane, chloroform, and EA) of a powdered alkaline ANE (Acadian; Acadian Seaplants Ltd) were utilized. Three-week-old plants grown on Jiffy-7 pellets under greenhouse conditions were irrigated with 20 mL of the aqueous solution and organic extracts (1 g L^{-1}) and 48 h later the temperature was lowered by 1 °C per day until the desired low temperature. Two days after the plants had been returned to the regular temperature regime (22 °C/18 °C day/night), the freezing damage was scored by means of diverse approaches. In the initial study (Rayirath et al. 2009), the degree of chlorosis and leaf damage was visually evaluated, the chlorophyll content and electrolyte leakage were measured, the macroscopic tissue damage was visualized by Trypan Blue staining, and membrane integrity was assessed with Nile Red staining via fluorescence microscopy. Treatment with the ANE, but especially with the lipophilic component-containing EA fraction induced systemic physiological responses that provided a considerable protection against freezing damage at the whole plant level, namely viability at −4.5 °C was increased by 40–60%, the damaged tissue area was reduced by 30–40%, the plasma membrane integrity and tissue organization were maintained, and the temperature that caused 50% electrolyte leakage was lowered by 3 °C. A two-step RT-PCR revealed that the threefold higher chlorophyll content in the ANE-treated plants correlated with a reduced expression of two chlorophyllase genes (*AtCLH1* and *AtCLH2*), involved in chlorophyll degradation. Additionally, by means of a RT-qPCR analysis, a twofold upregulation was detected of the transcription factor *DREB1A/CBF3* and its target cold response genes *COR15A* encoding a chloroplast stromal protein with cryoprotective activity and *COR78/RD29A*, a key regulator of drought, salinity, and low temperature. This signal transduction cascade could possibly actively induce downstream genes implemented in freezing tolerance.

The follow-up study revealed an important role for osmoprotectants, such as proline and soluble sugars, in the improved freezing tolerance of ANE-treated plants (Nair et al. 2012). ANE application and particularly of its lipophilic fraction resulted in the accumulation of proline and soluble sugars, but could not protect the proline biosynthesis-deficient mutant *p5cs-1* and the sugar accumulation-defective mutant *sfr4* against freezing damage. Metabolite analysis with two-dimensional nuclear magnetic resonance confirmed the increase in sugar and sugar alcohols in plants treated with the lipophilic ANE fraction prior to freezing and, additionally, indicated an accumulation of unsaturated fatty acids, possibly related to the altered membrane fluidity that enhances membrane integrity and cellular function during freezing. The subsequent assessment of global transcriptional changes (≥1.5-fold) elicited by the lipophilic ANE fraction by means of the GeneChip ATH1 Affimetrix microarrays revealed that approximately 5% (1113 genes) of the genome was affected in plants harvested during freezing stress and only 1.65% (398 genes) after freezing treatment, with a small overlap between both sets of transcripts. Overall, the trend in the different functional up- and downregulated classes during and after freezing was opposite, suggesting a specific ANE mode of action during freezing stress. The accumulation of proline was supported by the upregulation of the proline biosynthesis genes *P5CS1* and *P5CS2* and the downregulation of the proline degradation gene *ProDH*. Furthermore, the increased levels of soluble sugars seemed to be achieved by several mechanisms, including the upregulation of polysaccharide degradation genes (such as *SEX1* and *SEX4/DSP4* and *MUR4*, involved in starch and galactose degradation, respectively), and soluble carbohydrate biosynthesis (glucose, fructose, raffinose/stachyose, such as *GOLS2* and *GOLS3*) and the downregulation of pathways involved in sucrose degradation (*At1g12240*). In agreement with the metabolome data, also the lipid metabolism was among the major pathways that were affected by the ANE treatment during freezing, such as the upregulation of the digalactosyldiacylglycerol synthase-encoding gene *DGD1* involved in galactolipid biosynthesis. Additionally, 40 differentially expressed genes were induced, related to low temperature stress tolerance, osmotic stress, biotic stress and balance control of diverse hormones, namely the genes coding for salicylic acid (SA) (*At1g18870*), spermine/spermidine biosynthesis (*At5g15950*), and cytokinin conjugation (*UGT73B2*, *UGT76C1/2*, *At2g43820*, and *At1g24100*), whereas the *GA2ox1* gene involved in gibberellin inactivation was repressed.

Zamani-Babgohari et al. (2019) tested the effect of ANEs in mitigating freezing stress in *Nicotiana tabacum* L. (tobacco) cv. Bright Yellow-2 (BY-2) suspension cells. BY-2 cells grown at 27 °C in the presence of the alkaline-extracted ANE Acadian (Acadian Seaplants Ltd), at concentrations of 0, 0.01, 0.05, and 0.1 g L^{-1} were subjected to 0, −3, or − 5 °C conditions for 24 h and then allowed to recover. Importantly, when no freezing stress condition was imposed, no positive effect on the biomass was observed. In contrast, at the highest concentration (0.1 g L^{-1}), the biomass was even significantly reduced. However, under freezing stress the DW of ANE-treated cells had increased after 3 days of recovery, although extensive variations in the measurements and the levels of obtained growth, it was noted to be up to 5 times smaller than in the control experiment without freezing stress. Furthermore, for the −5 °C condition, the cell viability was estimated to be 77% for cultures treated with 0.1 g L^{-1} ANE, whereas it was less than 20% in the control group. In cells treated with 0.1 g L^{-1} ANE the ion leakage was 37% after recovery at day 7, and 53 and 65% when treated with 0.05 and 0.01 g L^{-1}, respectively. qRT-PCR analysis showed a lower expression of Activating protein 2, betaine aldehyde dehydrogenase (*BADH*), glutathione *S*-transferase, and fucosyltransferase in ANE-treated cells compared with the control, indicating that these cells experience less stress than under the control condition. In contrast, the expression of galactinol synthase 2 was upregulated under freezing stress in the ANE-treated groups, indicative of an enhanced defense against stress, whereas *DGD1* was upregulated only after 6 h of recovery in the control plants and pyrroline 5-carboxylate synthase (*P5CS*), involved in proline biosynthesis, was induced 2 and 6 h after cold stress in ANE-treated and control plants, respectively. The acetyl-CoA carboxylase gene was induced early in cultures under ANE treatment (after 4 h), but it was also upregulated after two additional hours of recovery in the control group. For the sake of comparison (cf. Rayirath et al. 2009 and Nair et al. 2012), it would have been interesting to investigate whether the lipophilic fraction of the ANE might have been efficient in mitigating cold stress too in this experimental setup.

Altogether, these studies illustrated that the ANE-induced physiological changes lead to protection against freezing damage and provided a foundation to the underlying molecular basis, but without functional analyses, it is difficult to draw strong conclusions from these data. Additionally, although both the aqueous and lipophilic fraction clearly contain components responsible for these changes, the identity of the elicitors and the subsequent signal transduction response still await elucidation.

Molecular analysis of ANE-induced drought stress tolerance in *Arabidopsis* and crops

To investigate the molecular basis of the positive impact of ANE applications on the photosynthetic performance during drought stress Santaniello et al. (2017) grew *Arabidopsis* Col-0 plants in a hydroponics system. After 20 days the plants were treated for 5 days with 3 g L^{-1} of ANE (soluble acidic

extract powder provided by Algea, Kristiansund, Norway), where after they were removed from the hydroponic solution and placed on filter paper for 4 days to induce water stress. Several physiological parameters were measured during the dehydration period, including the relative foliar water content, gas exchange and chlorophyll fluorescence. At the same time the expression of 14 relevant marker genes involved in these processes was determined by real-time qPCR. Whereas 90% of the untreated plants died within 4 days of treatment, almost all ANE-treated plants survived, maintained a 90% relative water content, and exhibited an improved water use efficiency. Interestingly, even prior to the water stress application, the ANE treatment resulted in a partial stomatal closure, decreasing the stomatal conduction by 55% and the transpiration rate by 53%. An ANE pretreatment also an overall reduced expression of *NCED3* (*At3g14440*), involved in ABA biosynthesis, and of *MYB60* (*At1g08810*), a transcription factor involved in stomata regulation, and an increased expression of the ABA-responsive genes *RAB18* (*At5g66400*) and *RD29A* (*At5g52310*). Altogether, these data indicate that an ANE pretreatment primed the plants for an improved survival. Indeed, after 2 to 3 days after the water stress imposition, the expression of *NCED3*, *RAB18*, and *RD29A* was highly induced in the absence of ANE. In contrast, due to the ANE pretreatment, the expression of the four marker genes remained largely unchanged. Whereas the photosynthetic CO_2 uptake was not modified by the ANE pretreatment, the intercellular CO_2 concentration was reduced. Upon water stress, the CO_2 assimilation rate and the mesophyll CO_2 conduction in untreated plants decreased sharply from 3 days onward, together with a significant increase in the intercellular CO_2 concentration. These processes were accompanied by a decrease in the gene expression of the photosynthesis-related *RBCS1A* (*At1g67090*) and *RCA* (*At2g39730*) and of *PIP1;2* (*At2g45960*) and *βCA1* (*At3g01500*) that are implicated in the regulation of CO_2 diffusion in the mesophyll. These physiological and molecular responses were strongly attenuated in the ANE-treated plants, suggesting that the drought-induced damage to the photosynthetic apparatus was prevented. In line with these findings, the ANE pretreatment resulted in the maintenance of a nearly optimal potential efficiency of the photosystem II (PSII) photochemistry (F_v/F_m) and an enhanced nonphotochemical quenching. Additionally, an increased expression of *PsbS* (*At1g44575*) and *VDE* (*At1g08550*) in the ANE-treated plants during dehydration hinted at an efficient energy dissipation and enhanced *DRF* (*At5g42800*) and *SOD* (*At1g8830*) expression at the activation of the antioxidant defense system that prevented oxidative damage to PSII. Although the modification of molecular pathways associated with improved drought tolerance upon ANE treatment has been demonstrated (Santaniello et al. 2017), as long as the ANE metabolites triggering these changes are not identified, the exact mechanisms involved remain elusive. The microarray analysis carried out by Goñi et al. (2016) to unravel the underlying molecular basis of ANE-induced plant growth stimulation in *Arabidopsis* had revealed an upregulation of the glutaredoxin family genes (*At1g0320* and *GRXC2*) and the cold-regulated gene *COR15A*. In agreement with the priming effect uncovered by Santaniello et al. (2017), ANE A and ANE B probably affected the enzymatic antioxidant system in plants, preparing them for future abiotic stress conditions.

ANE treatment-improved drought tolerance was also reported in soybean (Shukla et al. 2017). Germinated soybean 'Savana' plants grown in an environmental chamber were pretreated for 3 weeks with 7 mL L^{-1} Acadian (two applications of 100 mL in the first 2 weeks and a final application until soil saturation in the third week), where after the irrigation was stopped. Three stages were analyzed: before stress (22 h after soil saturation), during stress (75 h after soil saturation), and during recovery (89 h after soil saturation, the plants were irrigated and analysis was done 8 h later). The ANE application resulted in a reduced wilting during the drought stress and in an enhanced recovery ability. Additionally, both under the drought conditions and during recovery, the ANE-treated plants exhibited a 46% higher stomatal conductance and a 20–27% higher reactive oxygen scavenging activity than control plants. Real-time qPCR of drought-associated marker genes supported a role for ABA in the improved stomatal conductance. Indeed, the expression of the *GmCYP707A1a* and *GmCYP707A3b* genes, both involved in ABA catabolism, was induced during drought stress and in the recovery phase, respectively. Furthermore, the expression of the ABA-inducible *GmDREB1B* and the BURP domain protein-encoding *GmRD22* had increased especially during drought stress, whereas the expression of the ABA-independent stress-responsive gene *GmRD20* remained unaltered. Similarly, the induced expression of the ABA-responsive fibrillin gene *FIB1a*, both during and after drought imposition, was in line with the improved photoprotection and the increased expression of the aquaporin gene *GmPIP1b* during the rehydration phase in the ANE-treated plants was correlated with the internal water movement maintenance and the consequently improved recovery. Besides the modified expression of these ABA-related genes, the ANE treatment also induced the expression of the glutathione S-transferase gene *GmGST*, the molecular chaperone *GmBIP*, and the antiquitin-like *GmTP55*, all implicated in reactive oxygen species detoxification. In contrast to the findings in *Arabidopsis* (Santaniello et al. 2017), no evidence for a priming effect of the ANE pretreatment was obtained in soybean (Shukla et al. 2017). Noteworthy, in both studies, the nature of the extracts used was different (soluble extract powder prepared at an acidic pH (Algea) vs alkaline-extracted Acadian), as was the experimental setup

(hydroponics vs soil) and the ANE concentration (3 g L^{-1} vs 0.5 g L^{-1}), making comparisons problematic.

ANE-mediated drought stress alleviation in tomato plants has been studied with three different ANEs: ANE A was extracted at neutral pH, whereas ANE B and ANE C were alkaline extracts (Goñi et al. 2018). All assessed components of their chemical composition (such as solids, ash, sulfate, uronic acid, fucose, polyphenol, laminarin, and mannitol) varied significantly. The amount of unidentified organic components was below 20% for ANE A and ANE B, but close to 30% for ANE C. For the experimental setup, 35-day-old tomato plants cv. Moneymaker were used. The first sampling of leaf tissue was before the first ANE application (T0) and 24 h after spraying with 0.33% ANE, drought stress was induced by withholding water for 7 days, whereafter the leaf tissue was sampled (T1). For the 2-week recovery phase, plants were rewatered and 24 h later the ANE treatment was applied for the second time, followed by the third sampling after 48 h (T2). The final leaf sample was taken at the end of the recovery stage (T3). At the end of the growth period, the effect of the ANEs on the alleviation of drought stress was assessed. ANE A and ANE C increased the FW and DW by 25 to 30%, but plants treated with ANE B were almost identical in size when compared with the untreated stressed control plants. Lipid peroxidation was assessed with malondialdehyde (MDA), a lipid peroxidation marker. Under drought stress the MDA content was approximately 30% lower in plants treated with ANE B and ANE C than that in the control, whereas this decrease was slightly smaller, but still significantly different from the control, with ANE A. For the different time points, the chlorophyll content was the highest with ANE A at T1 and T3, whereas the same chlorophyll level as the control was attained with ANE C over the different time points and even decreased by 7.5% at T2. Additionally, the amount of proline, glucose, and sucrose also significantly increased under drought stress (T1) with the ANE A treatment and, in the post-drought stress period (T2), the proline level in the plants treated with ANE A and ANE C was higher than that in the control. Finally, molecular data were obtained on the impact of the ANE treatment on plant dehydrins, which are important players in adaptation to abiotic stress, using a polyclonal serum raised against the K segment of the dehydrins and qRT-PCR analysis of the dehydrin *tas14* gene. With the serum eight different polypeptide bands ranging from 15 kDa to 38 kDa were recognized and only ANE A treatment increased the 32, 18 and 15 kDa dehydrin levels in drought-stressed plants. The expression of the dehydrin *tas14* gene on the other hand was upregulated by all ANEs, but whereas ANE A treatment resulted in an 8-fold increase, plants treated with ANE B and ANE C only exhibited a little over 2-fold induction.

Altogether, these studies illustrate the difficulties of aligning phenotypical data with the composition of ANEs, which is aggravated by the fact that a relatively large amount of components, which may or may not play a role in the observed phenotypes, cannot be characterized. Furthermore, even though some ANEs may show a very similar phenotype, such as drought stress alleviation, the specifics of the stress-related parameters and, hence, the underlying mechanisms, may differ. Likewise, although some of the observed stress-related parameters may have similar values, the phenotypical outcome may be very different.

Molecular analysis of ANE-induced salinity stress tolerance in *Arabidopsis*

Based on undisclosed results of a microarray analysis of *Arabidopsis* Col-0 plants subjected to 125 mM NaCl in the presence or absence of the organic fraction of an alkaline ANE, genes not previously linked to salinity stress tolerance, of which the expression was downregulated in the presence of ANEs, were functionally analyzed by means of knockout mutants. As such, *At1g62760*, encoding a putative pectin methylesterase inhibitor, was identified as a novel negative regulator of salt tolerance (Jithesh et al. 2012). In a subsequent study, 2-week-old *Arabidopsis* Col-0 plants subjected to 100 and 150 mM NaCl for 24 h were treated with a 1 g L^{-1} equivalent of a methanolic ANE fraction (MEA) (Jithesh et al. 2018). As compared with the control, the plants treated with 150 mM NaCl and MEA had a larger leaf area (+37.3%), an increased plant height (+33%), more leaves (+33%), and a higher biomass (+57%), whereas the increases were slightly lower in the plants treated with 100 mM NaCl and MEA. The methanolic fraction was further subfractionated into water-soluble, chloroform, and EA fractions. Again when compared with the control, plants treated with 150 mM NaCl and EA had a larger leaf area (+ 62%), an increased plant height (+ 48%), a higher number of leaves (+ 45%), and an increased biomass (+ 52%). As EA mitigated the NaCl stress better than any other subfraction, a microarray analysis was conducted with plants treated with this extract. In the EA treatment, 184 and 257 genes were upregulated and 91 and 262 genes were downregulated on day 1 and day 5, respectively. On day 1, the transcripts for late embryogenesis abundant 3 family (*LEA3*, *At1g02820*) and the transcription factor *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*, *At2g46830*) were the most strongly induced. The finding that *LEA3* and *LEA1* (*At5g06760*) were induced upon treatment is in agreement with the results of Goñi et al. (2016). *CCA1* and by extension the circadian rhythm mechanism in plants has been linked before to abiotic stress (Grundy et al. 2015). Additionally, dehydration-responsive or DRE-binding proteins were also induced, as well as the glutathione *S*-transferase-encoding gene *At5g172200*. Downregulated transcripts included cellulose synthase (*At1g55850*), the auxin-responsive gene *At2g23170*, the pectin methylesterase inhibitor protein

At1g62760 and several heat shock proteins. Also on day 5, transcripts of *LEA1* and the *LEA2* group were induced. Additionally, the genes *AtHVA22b* (*At5g62490*) and *Di21* (*At4g15910*), involved in ABA signaling, were upregulated. Furthermore, several lipid transfer proteins (LTPs), which are known to play a role in (a)biotic stress(es), were also induced. (Xu et al. 2018). Downregulated genes included *WAK1*, pectin esterases (*At1g53840* and At3g10720), and pyruvate dehydrogenase (*PDH*, *At3g30755*), the latter playing a role in proline accumulation under osmotic stress.

The putative role of micro RNAs (miRNAs) in the regulation of ANE-triggered changes during salinity stress has also been studied (Shukla et al. 2018). miRNAs silence genes postranscriptionally by targeting mRNA degradation or by interfering with translation and their expression is often induced by various abiotic stresses (Shriram et al. 2016). *Arabidopsis* Col-0 plants were grown on Jiffy-7 W peat pellets for 3 weeks prior to the application of four conditions (20 mL per plant, once a week for 3 weeks): water (irrigated control), ANE treatment, NaCl-supplemented ANE treatment, and NaCl treatment. The ANE used was the commercially available Acadian Marine Plant Extract Powder (a KOH extract) prepared at 0.1% (*w/v*). The salinity stress was achieved with 150 mM NaCl. The ANE-treated plants under saline stress had higher FW and DW than plants subjected to saline stress without ANEs, illustrating an improved stress tolerance. After 6 and 12 h post treatment, small RNAs were isolated and sequenced via the Illumina platform, revealing that 106 miRNAs were differentially expressed in at least one of the treatments and/or time points. Overall, for several miRNAs, the ANE treatment seemingly attenuated the salinity stress-triggered induction level. Putative in silico predicted targets of the miRNAs modulated by the ANE treatment or by the ANE-mediated salinity tolerance included transcription factors and other genes involved in very diverse biological processes. For 12 salinity stress-modulated miRNAs, the expression of 14 of their putative target genes could (partially) explain particular processes implicated in this stress mitigation. For instance, the ANE treatment in the presence of NaCl enhanced the expression of *ath-miR396a-p* that downregulates the *AtGRF7* transcription factor, which, in turn, resulted in an increased expression of *AtDREB2a* and *AtRD29*, thus contributing to salinity tolerance enhancement. In contrast, the expression of *ath-miR169-5p* was transiently repressed by the combined treatment of ANE and NaCl, increasing the expression of its target transcription factors *AtNFYA1* and *AtNFYA2*, with an improved stress tolerance as a result. Interestingly, ANE-treated plants had a lower Na^+ and a higher P content in the presence of NaCl than plants treated with NaCl alone, implying that the ANE treatment can prevent the phosphate uptake impairment typically associated with drought and salinity. The NaCl-induced expression level of *ath-miR399a*, *ath-miR399b*, *ath-miR399c-3p*, and *ath-miR399c-5p* that are involved in the regulation of phosphate starvation responses was strongly attenuated by the ANE treatment, leading to an increased expression of the target genes *AtUBC24* and *AtWAK2*. Similarly, the high expression of *at-miR827* in the presence of NaCl was reduced by the ANE treatment, also increasing the expression of its target *AtNLA* gene under these circumstances. These studies demonstrated that the alleviation of salt stress when different ANEs are applied results from the contribution of a multitude of different stress-related genes (Jithesh et al. 2018) and involves phosphate homeostasis (Shukla et al. 2018).

Molecular analysis of ANE-induced biotic stress resilience in *Arabidopsis* and crops

For most physiological effects induced by seaweed extracts, the bioactive molecules responsible for the initiation of the responses are unknown. In contrast, some of the elicitors have been identified that trigger biotic stress tolerance in a wide variety of plants (Vera et al. 2011). Indeed, especially the major cell wall polysaccharides of brown algae such as alginates and fucans, the principal storage polysaccharide laminarin, and their derived oligosaccharides have been shown to induce an oxidative burst and defense signaling pathways mediated by SA and jasmonic acid (JA)/ethylene (Eth) in plants. In turn, these responses result in the accumulation of antimicrobial pathogenesis-related proteins, defense enzymes, and phenolics that culminate in enhanced protection against a broad range of pathogens. Nonetheless, the specific receptors of these elicitors remain to be determined and, to our knowledge, genome-wide analyses to get a holistic view on the molecular basis of ANE-induced biotic stress resistance have not been reported yet.

The effect of ANE treatments was analyzed on disease development imposed by the hemi-biotrophic *Pseudomonas syringae* pv. *tomato* DC300 (*Pst* DC300), the causative agent of bacterial speck, and on the expression of defense markers in *Arabidopsis* (Subramanian et al. 2011). Wild-type Col-0 plants and mutants in the SA and JA/Eth defense pathways were grown in Jiffy-7 peat pellets for 3 weeks in a growth chamber and irrigated with different ANE solutions (25 mL of 1 g L^{-1} solutions). In total, three different extracts were used: an aqueous solution and two organic subfractions (sequential fractionation of the MeOH fraction with chloroform and EA). After 48 h of ANE treatment fully expanded leaves were pressure inoculated with the hemi-biotrophic *Pst* DC300. All ANE treatments restricted the *Pst* DC300-induced symptoms to minor chlorosis at the inoculation site and significantly reduced disease severity; the strongest reduction was observed with the EA extract (35 vs 57% of the control disease severity). The bacterial titers in the ANE-treated plants were lower than those of the untreated controls.

Nevertheless, a direct antimicrobial effect was ruled out, because the ANEs themselves stimulated bacterial proliferation under culture conditions. *Pst* DC300 resistance involves SA-mediated defense pathways (Uppalapati et al. 2007), but analysis of the responses of ANE-treated *NahG* transgenic plants and *ics1* mutants that do not accumulate or produce SA, respectively, revealed the same disease reduction level as in wild-type plants. The ANE treatment seemingly did not affect the expression of the SA-inducible *PR1* gene as analyzed in 7-day-old *PR1::GUS* plants grown in vitro and treated with the ANEs for 48 h in liquid medium. RT-qPCR revealed that the expressions of *PR1* and more strongly of *ICS1* were repressed in ANE-treated compared with untreated plants, a downregulation speculatively attributed to laminaran (Mercier et al. 2001). Whereas the *Pst* DC300 infection did not result in an upregulation of these genes in the control plants, the *ICS1* expression was moderately and that of *PR1* strongly induced, especially in combination with the aqueous ANE treatment. Based on these data, the SA-mediated systemic acquired resistance was considered not to be involved in the ANE-induced protection of *Arabidopsis* against *Pst* DC300. Concerning the JA/Eth pathway, the ANE treatment of *jar1* mutant plants, defective in the formation of a biologically active jasmonyl-isoleucine (JA-Ile) conjugate, did not reduce the disease severity or the bacterial titers compared with control plants. GUS staining of ANE-treated *pAOS::GUS* plants and RT-qPCR showed no differential expression of this allene oxide synthase gene implicated in JA-dependent signaling. In contrast, the expression of the JA-responsive defensin *PDF1–2* was activated especially by treatment with the chloroform subfraction and, when combined with *Pst* DC300 infection, the *PDF1–2* expression was even further increased. Together these data suggested that the lipophilic ANE components primed the JA-mediated defense responses and that these defense mechanisms were essential for the ANE-induced systemic resistance. Additionally, simultaneously with the root treatment, shoots received an extra 2 mL spraying with the aqueous ANE and 48 h later the leaves were either inoculated with mycelial plugs of the necrotrophic white mold-causing *Sclerotinia sclerotiorum* or with its pathogenicity factor oxalic acid. The ANE treatment increased the resistance against *S. sclerotiorum* as evidenced by the delayed and reduced size of the lesions formed, most probably by suppression of the oxalic acid-induced toxicity. Although no further molecular data were provided, the presence of sterols and fatty acids in the ANE was hypothesized to activate nonspecific lipid transfer proteins in the plasma membrane involved in JA-mediated disease signaling cascades (Subramanian et al. 2011).

A putative priming effect of biotic stress tolerance by ANEs was also detected in the microarray analyses of Goñi et al. (2016). Indeed, the ANE treatment of *Arabidopsis* activated *PR1*, *PR5*, and WRKY transcription factor-encoding genes, indicating that the SA pathway is enhanced, whereas upregulation of two putative 2-oxoglutarate-dependent dioxygenases (*At5g43450* and *At2g25450*) and ethylene-responsive transcription factors (*ERF2* and *ERF72*) hinted at the JA/Eth pathway activation.

The protective effect of ANE pretreatments against the fungal pathogens *Alternaria radicina* and *Botrytis cinerea* (Jayaraj et al. 2008), causing black rot and gray mold, respectively, was also analyzed in carrot, either as such or combined with a single fungicide treatment (2 g L^{-1} chlorothalonil-50%). Eight-week-old carrot "High Carotene Mass" plants grown in a greenhouse were sprayed with a 0.2% aqueous ANE solution (Acadian Seaplants Ltd) until runoff; after 12 h, samples were taken and then daily until 4 days after treatment for the analysis of the expression and activity of defense-related enzymes. Northern blot analyses revealed that the expression of the genes *PR1*, *PR5*, SA receptor *NPR-1*, lipid transfer protein (*LTP*), chitinase (*U52848*), chalcone synthase-2 (*CHS-2*), and *PAL-1* were all upregulated from 12 h after treatment until 72 h where after the expression decreased. In agreement with the expression data and following similar kinetics, the chitinase and PAL activities were higher in ANE-treated plants than in control plants as well as the peroxidase, polyphenoloxidase, β-1,3-glucanase, and lipoxygenase (LOX) activities. In line with the enhanced activities of PAL, peroxidase, and polyphenoloxidase, the total phenolic content of the ANE-treated plants had increased. The improved LOX activity was correlated with a clear H_2O_2 accumulation in response to the ANE treatment. Interestingly, similar molecular and biochemical responses were obtained by treatment with 100 μM SA, but overall the effect of the ANE treatment was stronger and sustained longer in time. For the analysis of the biotic stress resilience, 6 h after the ANE treatment, the carrot plants were inoculated with conidial suspensions of the two fungal pathogens. The ANE treatment was repeated 10 and 20 days after infection and disease development was analyzed after 10 and 25 days. Disease development for both pathogens was already reduced by a single ANE treatment, but additional ANE applications resulted in an even lessened disease incidence. Importantly, the ANE treatment provided better protection than treatments with SA or fungicides, but the combined application of a single ANE and a single fungicide spray gave the best disease suppression. Additionally, the ANE-treated plants had the highest biomass, but their nutrient composition was unaltered compared with the other treatments. Thus, the ANE-induced resilience of carrots to two fungal pathogens was seemingly accomplished by SA-mediated systemic acquired resistance, possibly triggered by the oligosaccharide fraction and other unidentified elicitors of the ANE (Jayaraj et al. 2008).

The effect of pretreatment with the commercial Stimplex was examined on disease development in cucumber after inoculation with either *Alternaria cucumerinum*, *Didymella applanata*, *Fusarium oxysporum*, and *Botrytis cinerea*¸ the

causing agents of *Alternaria* blight, Gummy stem blight, *Fusarium* root and stem rot, and *Botrytis* blight, respectively (Jayaraman et al. 2011). Cucumber var. *sativus* plants were grown for 30–40 days in a greenhouse before they were either sprayed, drenched, or sprayed and drenched with 0.5 or 1% ANE solutions. After 12 h and then daily for 4 days, leaf samples were taken for transcript and enzyme activity analyses. As assessed by Northern blot analysis, the expression of a chitinase, *LOX*, glucanase, peroxidase, and *PAL* genes were all significantly upregulated by the ANE treatments, but the strongest induction for all time points tested was obtained by the combined spraying and drenching. In agreement with the expression data, the chitinase, LOX, glucanase, peroxidase, and PAL activities were the highest in the sprayed and drenched plants, as well as the polyphenoloxidase activity. Additionally, in correlation with the increased PAL activity, the total phenolic content in sprayed and drenched plants was higher than that in control plants. For the effect on symptom development, the initial ANE treatment was repeated twice with 10-day intervals. Then, 6 h after the last ANE treatment, the plants were inoculated with conidial suspensions of the fungi. Additionally, spraying with ANE was combined with a single fungicide treatment (2 g L^{-1} chlorothalonil-50%) applied 10 days after pathogen infection. For all fungal pathogens, the best disease reduction was obtained with the combined spraying and drenching treatment with 0.5% ANE (70% for *Alternaria*, 47% for *Didymella*, 46% for *Botrytis*, and 88% for *Fusarium*), but an additional application of the fungicide provided an even better result (75% for *Alternaria*, 60% for *Didymella*, 55% for *Botrytis*). In the case of *Fusarium*, the fungicide alone already prevented disease development altogether, but the combination with the ANE treatment resulted in increased shoot and root biomasses. Thus, both for carrot and cucumber, comparable results were obtained.

With the same experimental setup (and astonishingly in nearly the exact same wording), Abkhoo and Sabbagh (2016) analyzed the impact of pretreatment with the commercial ANE Marmarine (International Ferti Technology Corporation, Amman, Jordan) on disease induction by *Phytophthora melonis*, another damping-off pathogen. Cucumber 'Negin' F1 plants grown for 21 days in a greenhouse were treated by spraying and drenching with 30 mL of Marmarine (0.5 or 1%). Every 24 h for four consecutive days, leaves were sampled for expression analysis and roots for enzyme activity determination. As revealed by qRT-PCR, the expression of the pathogen-induced *Cupi4*, *LOX*, *PAL*, and galactinol synthase *GolS* genes was activated by all ANE treatments, but the strongest in sprayed and drenched plants. Overall, the expression peaked between 48 and 72 h after Marmarine treatment. In accordance with Jayaraman et al. (2011), peroxidase, polyphenoloxidase and β-1,3-glucanase activities as well as the total phenolics content were also higher in the ANE-treated plants. For the pathogenicity tests, after the initial ANE drench treatment, two additional Marmarine treatments were applied with 5-day intervals and 6 h later the plants were inoculated with zoospore suspensions of *P. melonis*. Additionally, the combined ANE and fungicide treatment was evaluated by drenching the plants 6 days after pathogen inoculation with 2 g L^{-1} metalaxyl G5%. Similar to the findings of Jayaraman et al. (2011), the combined spraying and drenching with 0.5% Marmarine resulted in the highest disease reduction (68%) and, although a single fungicide treatment reduced disease incidence even further (close to 80%), the combination with the Marmarine treatment yielded an increased root biomass.

Concluding remarks and perspectives

As an entry point to get insight into the impact of ANEs on plant growth and stress tolerance, different approaches have been combined, including the evaluation of morphological modifications (such as root and shoot growth or FW and DW), physiological responses (photosynthetic parameters), biochemical analyses (enzyme activities, hormone profiling, and phenolic composition), responsiveness of mutant lines, and/or gene-specific or genome-wide determination of mRNA levels. Overall, it has become increasingly clear that ANEs affect the endogenous balance of plant hormones by modulating the hormonal homeostasis, regulate the transcription of a few relevant transporters to alter nutrient uptake and assimilation, stimulate and protect photosynthesis, and dampen stress-induced responses.

Despite the progress made the exact molecular basis of improved growth and stress adaptation induced by ANE treatment proves difficult to unravel, partly because of the polygenic response implicated in such intricate and dynamic processes and of the complexity to discriminate between direct and secondary effects. For instance, the genome-wide transcriptomic analyses of ANE-induced plant growth promotion (Nair et al. 2012; Jannin et al. 2013; Goñi et al. 2016) illustrated that ANE treatments broadly redirect gene expression in plants, but at the same time that the differential gene sets show very little overlap. Additionally, the achievement of a general model on the ANE action mechanism has been impeded by (i) the diversity of the experimental setups used, such as the type and duration of the treatments and age and growth conditions of the plants, (ii) the use of different ANE products at different concentrations, (iii) the often incomplete compositional information, (iv) the poor reproducibility due to seasonal fluctuations in algal compositions, (v) the occurrence of species-dependent effects, and (vi) the complex relationship between ANE composition and biostimulant activity. Thus, understanding the ANE mode of action is imperative, not only from a fundamental point of view but also from a scientific viewpoint to support the biostimulant claim for future

registration of particular ANE products. Indeed, the increased interest in seaweeds as biostimulants impels the establishment of a regulatory framework.

To extend the current knowledge on the mechanisms of ANE action in different phenotypic responses, future research should focus on implementing fractionation techniques in new comprehensive experiments that assess the effect of the major and minor components of the ANE biostimulants singly and in combination, and transcriptome-wide and proteomic studies of ANE treatments should be directed toward the identification of marker genes for the beneficial responses that can be used in biostimulant product development (Calvo et al. 2014). Additionally, by focusing on the very early events during plant-microbe interactions, the putative receptors (and coreceptors) of and the associated signaling pathways triggered by particular bioactive ANE components should be identified to comprehend the simultaneous activation of plant growth and defense against pathogens in ANE-treated plants (González et al. 2013). Moreover, the potential involvement of posttranslational modifications should be explored to understand the observed effects of ANEs (Billard et al. 2014). Even more, the importance of cell elongation and cell proliferation in ANE-stimulated shoot and root growths should be dissected in depth by means of available phenotyping initiatives (Dhondt et al. 2013). And finally, the current lack of functional data should be alleviated with the use of mutants. All in all, despite significant efforts, the road toward the proposition of a comprehensive model on the molecular mode of action of ANE-induced responses is still long, but imperative for the increased credibility of ANEs as robust crop inputs.

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