Review

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Phytohormones in red seaweeds: a technical review of methods for analysis and a consideration of genomic data

DOI 10.1515/bot-2016-0056
Received 20 June, 2016; accepted 23 February, 2017; online first 30 March, 2017

Abstract: Emerging studies suggest that seaweeds contain phytohormones; however, their chemical entities, biosynthetic pathways, signal transduction mechanisms, and physiological roles are poorly understood. Until recently, it was difficult to conduct comprehensive analysis of phytohormones in seaweeds because of the interfering effects of cellular constituents on fine quantification. In this review, we discuss the details of the latest method allowing simultaneous profiling of multiple phytohormones in red seaweeds, while avoiding the effects of cellular factors. Recent studies have confirmed the presence of indole-3-acetic acid (IAA), N^6-(Δ2-isopentenyl)adenine (iP), (+)-abscisic acid (ABA), and salicylic acid, but not of gibberellins and jasmonate, in *Pyropia yezoensis* and *Bangia fuscopurpurea*. In addition, an *in silico* genome-wide homology search indicated that red seaweeds synthesize iP and ABA *via* pathways similar to those in terrestrial plants, although genes homologous to those involved in IAA biosynthesis in terrestrial plants were not found, suggesting the epiphytic origin of IAA. It is noteworthy that these seaweeds also lack homologues of known factors involved in the perception and signal transduction of IAA, iP, and ABA. Thus, the modes of action of these phytohormones in red seaweeds are unexpectedly dissimilar to those in terrestrial plants.

Keywords: epiphytes; genome-wide survey; hormone metabolism; liquid chromatography–mass spectrometry; phytohormone; red seaweed; simultaneous analysis.

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Abbreviations: ABA, (+)-abscisic acid; cZ, *cis*-zeatin; DMAPP, dimethylallyl diphosphate; FAB, fast atomic bombardment; IAA, indole-3-acetic acid; iP, *N*6-(2-isopentenyl)adenine; IPT, isopentenyltransferase; LOG, LONELY GUY; MEP, methylerythritol phosphate; MRM, multiple reaction monitoring; MVA, mevalonate; NCED, 9-*cis*-epoxycarotenoid dioxygenase; SIM, selected ion monitoring; SPE, solid-phase extraction; tZ, *trans*-zeatin; ZEP, zeaxanthin epimerase

Introduction

Phytohormones (also known as plant hormones) are signaling molecules biosynthesized in plants that regulate the physiological functions of plants. They control architecture, developmental stage, and stress responses and are – in principle – universal among plants. The active concentrations of phytohormones *in planta* are very low (10^{-10}–10^{-6} mol kg^{-1}, e.g. Table 1; Kamiya 2010). In animals, hormones are produced in specific parts of organs, secreted from specialized glands, and elicit responses in specific target organs (Dodds et al. 1956). By contrast, production of phytohormones is not restricted to specific tissues in the plant, and the target tissues are ubiquitous (Kamiya 2010). As well as being able to reach every part of an individual plant, some volatile hormones can even reach neighboring individuals (Pichersky and Gershenzon 2002). Plant cell responses to phytohormones are multiplex, reflecting the nature of plants as a dispersed biological information processing system in contrast to animals' centralized system (Kamiya 2010). Like plants, seaweeds do not have a central nervous system and are controlled by a dispersed system; however, the question remains whether seaweeds have plant hormones like their land-based relatives and, if so, how such phytohormones function in seaweeds.

Phytohormones include structurally unrelated compounds. The five so-called "classic" classes of

Table 1: Phytohormone contents of barley leaf blades, dry wheat seeds, and *Arabidopsis* shoots.

Phytohormones	Barley leaf blade (mol · kg^{-1})	Dry wheat seed (mol · kg^{-1})	*Arabidopsis* shoot (mol · kg^{-1})
Gibberellins			
Gibberellin A_1	$7.37 \pm 0.10 \cdot 10^{-10}$	Not detected	$2.68 \pm 0.25 \cdot 10^{-10}$
Gibberellin A_4	$1.43 \pm 0.17 \cdot 10^{-9}$	$3.52 \pm 1.35 \cdot 10^{-10}$	Not detected
Cytokinins			
trans-zeatin	$3.42 \pm 0.68 \cdot 10^{-10}$	$1.52 \pm 0.37 \cdot 10^{-9}$	$5.78 \pm 0.24 \cdot 10^{-10}$
Isopentenyladenine	$4.88 \pm 0.89 \cdot 10^{-9}$	Not detected	$4.37 \pm 1.37 \cdot 10^{-10}$
Auxin			
Indoleacetic acid	$3.00 \pm 0.37 \cdot 10^{-8}$	$4.21 \pm 0.56 \cdot 10^{-7}$	$4.45 \pm 0.53 \cdot 10^{-8}$
Abscisic acid	$2.24 \pm 0.36 \cdot 10^{-8}$	$9.13 \pm 1.02 \cdot 10^{-8}$	$2.10 \pm 0.79 \cdot 10^{-9}$
Jasmonates			
Jasmonic acid	$4.91 \pm 0.49 \cdot 10^{-8}$	$8.87 \pm 3.61 \cdot 10^{-8}$	$1.89 \pm 0.49 \cdot 10^{-8}$
Jasmonoyl-isoleucine	$2.72 \pm 0.79 \cdot 10^{-9}$	$1.61 \pm 0.69 \cdot 10^{-8}$	$6.90 \pm 6.49 \cdot 10^{-10}$
Salicylic acid	$1.29 \pm 0.98 \cdot 10^{-7}$	$1.04 \pm 0.35 \cdot 10^{-6}$	$7.27 \pm 2.79 \cdot 10^{-8}$

phytohormones are auxin, abscisic acid (ABA; Figure 1A), gibberellins (GAs), cytokinins, and ethylene. Other classes of phytohormones more recently described include jasmonates (JAs), salicylic acid (SA; Figure 1B), brassinosteroids, and strigolactones (Kende and Zeevaart 1997, Kamiya 2010). Some phytohormones are known to exist in seaweeds as in terrestrial plants (Basu et al. 2002, Le Bail et al. 2010, Wang et al. 2014a, Mikami et al. 2016). However, the functions of phytohormones in seaweed development and stress responses remain uncertain.

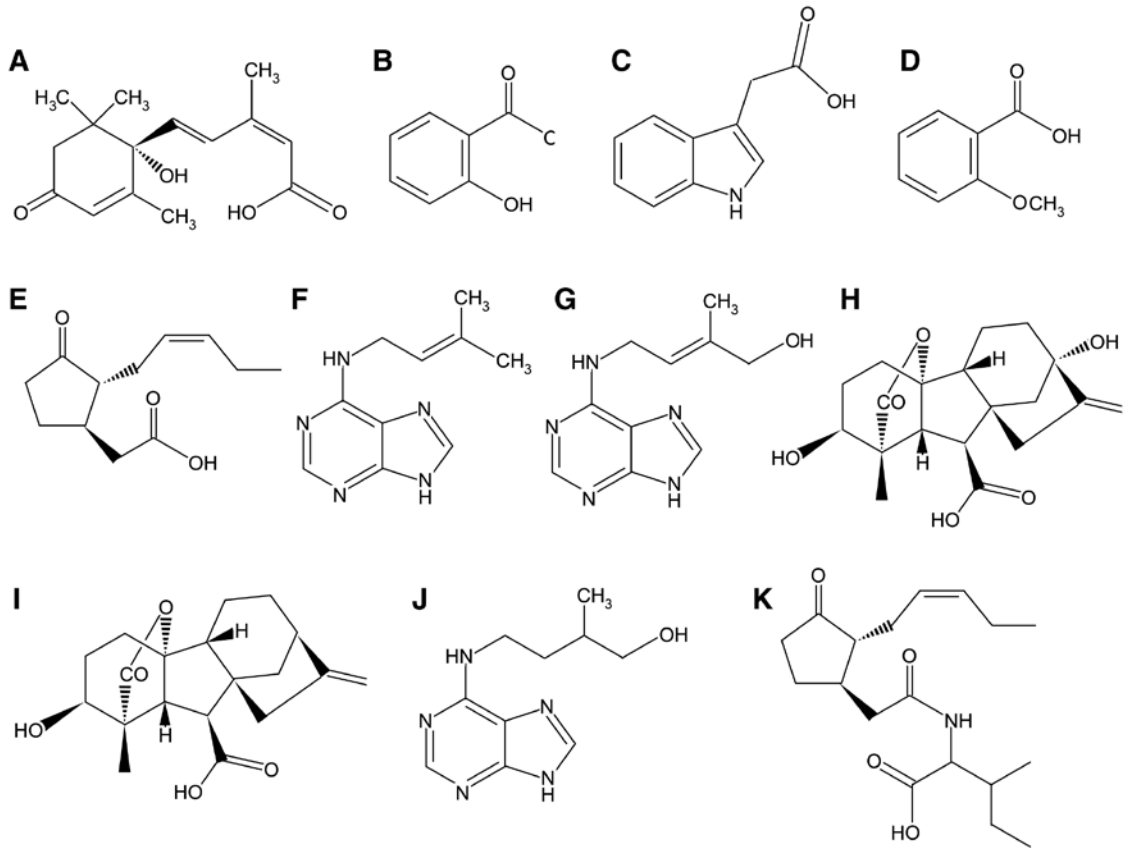

Figure 1: Structure of phytohormones.
(A) Abscisic acid, (B) salicylic acid, (C) indole–3–acetic acid, (D) *ortho*–anisic acid, (E) jasmonic acid, (F) isopentenyladenine, (G) *trans*–zeatin, (H) gibberellin A_1, (I) gibberellin A_4, (J) dihydrozeatin, (K) jasmonoyl-leucine.

Recent advances have revealed some roles of peptide hormones in plants in addition to the above-mentioned orthodox phytohormones (Matsubayashi and Sakagami 2006). Peptide hormones are processed from precursor peptides, unlike the orthodox phytohormones, which are synthesized by secondary metabolic pathways (Kamiya 2010). One can speculate that the evolutional history of peptide hormones from ancestral algae to terrestrial plants is distinct from that of the orthodox phytohormones in plants. Furthermore, analyses of peptide hormones in seaweeds have not been well established yet. In this article, we do not discuss peptide hormones further.

Polyamines, such as spermine, ptrescine, and spermidine, act as regulatory molecules in plant stress responses (Galston and Sawhney 1990, Alcazar et al. 2010). They interact with plant hormone signaling and a wide range of metabolic pathways (Alcazar et al. 2010). However, it seems that they have not yet been recognized as phytohormones, in general. Therefore, we will also not discuss polyamines in this article. In addition, we will not discuss ethylene, although it seems to be produced in red seaweeds (Watanabe and Kondo 1976, Garcia-Jimenez et al. 2013), because of the lack of knowledge about ethylene in algae to date.

Recent advances in techniques to analyze phytohormones using liquid chromatography–mass spectrometry (LC–MS) allow simultaneous analysis of multiple hormones for a comprehensive view of phytohormone dynamics (for example, Tokuda et al. 2013, Schäfer et al. 2016). Despite these advances, no comprehensive analysis of phytohormones has yet been carried out in red seaweeds. Collating data from comprehensive analyses of these hormones in seaweeds, as well as up-to-date genome-wide information, will aid in elucidating the functions of phytohormones in seaweeds, as well as the evolution of phytohormones in Plantae.

Despite their expected importance, only a very limited number of papers has been published in which comprehensive phytohormone analyses of algae were reported (Wang et al. 2014a,b, Mikami et al. 2016). We suggest that the main problems of phytohormone analysis in algae are (1) that the methodology developed for terrestrial plants may not be applicable to algae, especially the extraction and pretreatment steps, and (2) that the phytohormones detected are possibly synthesized not in algal cells, but in obligate epiphytic microorganisms. In this article, we review practical problems in methods of quantification of phytohormones in general and provide current genomic insights into hormone metabolism and signaling in red seaweeds.

Quantification of phytohormones

A brief review of methods used to analyze phytohormones in terrestrial plants

Phytohormones were originally discovered in terrestrial plants, and not in algae. Consequently, the methods of phytohormone analysis have been mainly developed for the tissues of terrestrial plants. Therefore, we first review phytohormone analysis methods for terrestrial plants. Several approaches have been utilized for quantifying phytohormones in terrestrial plants before LC–MS became available. Selected ion monitoring (SIM) by gas chromatography–mass spectrometry (GC–MS) is a sensitive and selective method to identify and quantify low molecular weight compounds. Therefore, it has been utilized to quantify phytohormones in terrestrial plants (e.g. Scott et al. 1982, Blechschmidt et al. 1984, Chen et al. 1988, Kamboj et al. 1999). For analysis by GC–MS, however, it is necessary to first purify target phytohormones by high-performance liquid chromatography (HPLC) and to derivatize some hormones, for example, by methylation or trimethylsilylation. Hence, it is tedious compared with LC–MS, as discussed below. The pros and cons of using GC–MS for identification and quantification of phytohormones are well documented (Hedden 1993). HPLC, coupled with a fluorescence detector, can be used to quantify active auxin, indole-3-acetic acid (IAA; Figure 1C), as it has intrinsic fluorescence (Crozier et al. 1980). The fluorescence detector allows the specific quantification of IAA in a crude tissue extract with high sensitivity. However, other methods for quantification of IAA (see below) have eclipsed this approach in most recent studies. A similar method can be used to quantify SA in plant extracts (Meuwly and Metraux 1993). In this case, methylsalicylate (also known as *ortho*-anisic acid; Figure 1D) can be utilized as an internal standard, hence this analytical method enables a high-precision analysis. It can also be beneficial for simultaneous quantification of the biosynthetic precursors cinnamic acid and benzoic acid, based on their characteristic fluorescence. Ultraviolet absorption and differential reflective index are often utilized for detection in HPLC systems in addition to the above-mentioned fluorescence detection. However, quantitative analysis using these techniques is not sufficiently selective or sensitive to quantify a phytohormone specifically. The fluorescence detection is only applicable to fluorescent substances. ABA and JA (Figure 1E) do not emit a specific fluorescence (Figure 2), and hence fluorescence detection is not applicable to analyze these two phytohormones.

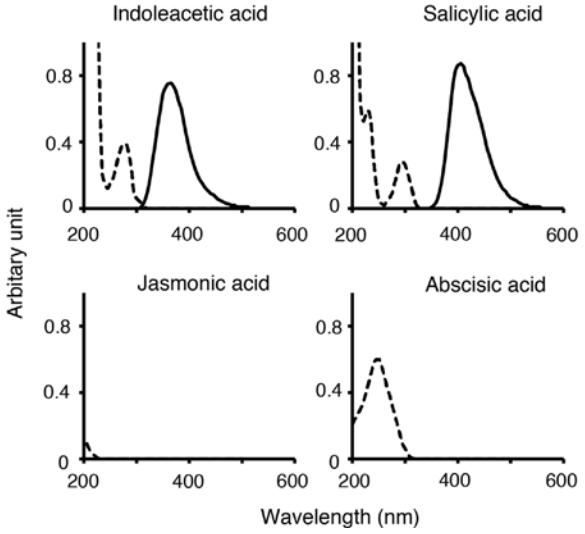

Figure 2: Absorption and fluorescence emission spectra of indole-3-acetic acid, salicylic acid, jasmonic acid, and abscisic acid.
Dotted and solid lines indicate absorbance spectra and fluorescence emission spectra, respectively. Excitation wavelength was 280 nm for indole-3-acetic acid, 300 nm for salicylic acid, and 250 nm for abscisic acid. Because of the weak absorbance of jasmonic acid between 200 and 500 nm, jasmonic acid fluorescence was not examined. Abscisic acid showed no fluorescence between 300 and 600 nm; hence, the line is not visible.

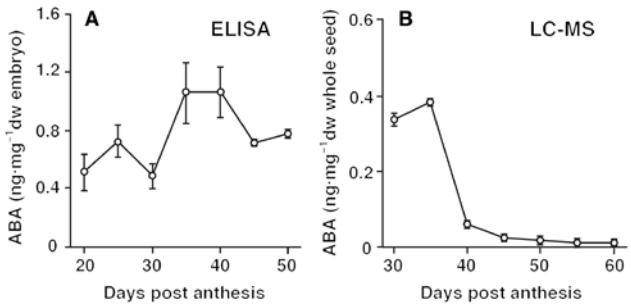

Figure 3: Dynamics of abscisic acid (ABA) content during seed maturation of the wheat cultivar Kitakei-1354, determined by two different quantification methods.
(A) Enzyme-linked immunoassay (ELISA) (modified from Suzuki et al. 2000). (B) Liquid chromatography-mass spectrometry (LC–MS). ABA content was determined according to Iehisa et al. (2013). Error bars represent standard deviations, n = 3. dw, dry weight.

Enzyme-linked immunoassay (ELISA) has been a standard choice for the quantitative analysis of ABA (Weiler 1982, Kawakami et al. 1997), but it has some drawbacks. It often has high background and shows a low signal-to-noise ratio, so that it has a lower sensitivity compared with GC–MS and LC–MS. ELISA captures the analyte of interest utilizing a specific immunoreaction of an antibody with the analyte. The immunoreaction between an analyte and an antibody can be interfered with by endogenous substances or polyreactive antibodies (Tate and Ward 2004). In fact, ELISA and LC–MS occasionally give totally different results. For example, the dynamics of ABA content during wheat seed maturation appeared different using ELISA compared with LC–MS (Figure 3; Suzuki et al. 2000, this study). It is therefore difficult to conclude that ELISA is a reliable method to quantify ABA, but it does have the advantage of being a high-throughput analytical method. ELISA kits to quantify other phytohormones are commercially available. Frequent and diverse glycosylation, amino acid conjugation, and methylation events in phytohormones (Westfall et al. 2013, Ostrowski and Jakubowska 2014) may cause interference in the analysis by cross-reaction. Accordingly, results achieved using the ELISA method should be further validated with other methods.

Most recently, utilization of the most state-of-the-art LC–MS technique, such as ultra-high-performance liquid chromatography combined with high-resolution Orbitrap (for example, Q–Exactive-Orbitrap tandem mass spectrometer, Thermo Fisher Scientific Inc.) has become available. A metabolomics approach using such high-resolution LC–MS is a powerful tool to analyze phytohormones in terrestrial plants (e.g. Van Meulebroek et al. 2012, 2014). This technique may apply to phytohormone analysis in seaweeds.

Quantification of phytohormones by LC-MS

Recently, LC-MS has frequently been utilized to quantify phytohormones in terrestrial plant samples in preference to other methods. LC–MS allows the simultaneous quantification of several phytohormones of not only a single terrestrial plant but also a seaweed sample (e.g. Chiwocha et al. 2003, Forcat et al. 2008, Yoshimoto et al. 2009, Kanno et al. 2010, Müller and Munné-Bosch 2011, Mikami et al. 2016). However, old-fashioned LC–MS was not necessarily suitable for phytohormone analysis. Unlike GC–MS, it is important for LC–MS to process a large quantity of solvent at the interface between LC and MS. The “moving belt interface”, a classic interface connecting LC and MS, transfers analytes from the outlet of LC in atmospheric pressure to the ion source in vacuum by a belt (Stout and DaCunha 1985). During the transfer on the belt, the solvent is evaporated by heat. Its large size and low stability are problems (Lemiere 2001). The thermospray (TSP) interface invented in the 1980s allows ionization of nonvolatile analytes with subtle thermal decomposition (Blakey and Vestal 1983, Vestal

1984, Lemiere 2001). An advantage of TSP is its capacity for a large quantity of solvent, which is associated with greater resolution and a fast HPLC flow rate. Also during the 1980s, continuous-flow fast atomic bombardment (FAB) was employed for the interface/ionization (Barber et al. 1982, Caprioli and Fan 1986, Fenselau and Cotter 1987). FAB is able to ionize substances with a large molecular mass as well as nonvolatile compounds that are soluble in a liquid matrix. The FAB ionization method does not require heating and thus allows analysis of heat-labile compounds. A drawback of FAB, however, is that it is not ideal for substances with a small molecular mass (<500 mass-to-charge ratio, *m/z*) because of chemical noise raised from the matrix. Herein lies the problem: most phytohormones have a molecular mass with *m/z* between 100 and 400 (e.g. SA, *m/z* 137; IAA, *m/z* 176; iP, *m/z* 204; ABA, *m/z* 263).

The more recently developed electrospray ionization (ESI) is the most successful LC–MS interface (Bruins et al. 1987, Banerjee and Mazumdar 2012) that allows phytohormone contents to be analyzed with high selectivity, high sensitivity, and high precision. In this technique, the sample in the ion source is ionized at atmospheric pressure, where the HPLC outlet is connected to the nebulizer (Bruins et al. 1987). ESI can be used to analyze a wide range of substances, from small to large molecular mass. It also allows the ionization of low polarity compounds in such a way that Coulomb explosions can protonate or deprotonate the compound in the ion source; this is achieved when high voltage is applied to the sample solution, and the solution is vaporized by hot nebulizing gas (e.g. nitrogen gas at 300°C) to yield protonated cations ($[M+H]^+$) and/or deprotonated anions ($[M-H]^-$) with a low degree of decomposition. Multiple reaction monitoring (MRM) produces unique fragment ions that can be selectively monitored. The MRM method consists of specifying the parent mass of the compound for MS/MS fragmentation and then specifically monitoring for a single fragment ion. This characteristic allows the LC–MS to be sensitive and specific even in very complicated matrices.

A shortcoming of LC-ESI-MS is the matrix effect, a phenomenon whereby coexisting substances in the analyte solution interfere with the ionization efficiency of the analyte of interest to produce a larger or smaller signal than the true quantity. Ion suppression occurs when matrix components compete with the analyte in the sample droplet to become charged and gain access to the surface of the droplet in the ion source. Methods to reduce or cancel the matrix effect will be discussed below.

Pretreatment of samples

LC-MS is a highly selective and specific method, although it is very sensitive to matrix effects. Therefore, pretreatment to partially purify target compounds is generally required to mitigate the matrix effects. The workflow from extraction to LC-MS data analysis is shown in Figure 4. Advances in pretreatment methods are another critical improvement that allow analysts to quantify phytohormones more effectively. Classically, liquid–liquid extraction (LLE) has been used to pretreat LC–MS samples (Cantwell and Losier 2002). Phytohormones of interest are usually separated from unwanted hydrophilic substances by migration to an organic solvent phase in appropriate conditions (e.g. carboxylic compounds such as IAA, ABA, SA, JA and GAs migrate to a hydrophobic phase in acidic conditions). Removing unnecessary substances greatly increases the sensitivity of LC–MS by reducing the effect of ion suppression and reducing blurring of the analytical column caused by insufficient separation.

The disadvantages of LLE are that it consumes large amounts of solvent, it requires a relatively large-scale sample preparation to compare with solid-phase extraction (SPE), and it is difficult to process in a high-throughput manner. Ninety six-well plate-based LLE is also available for high-throughput extraction (Steinborner and Henion 1999). The development of convenient SPE filling materials has, however, made extraction of the samples quicker and more selective. Seo et al. (2011) and Mikami et al. (2016), for example, used three different SPE cartridges to extract and fractionate phytohormones in a stepwise manner (Figure 5), to sequentially analyze 10 hormones from single samples of terrestrial plants, and a seaweed (e.g. Figure 6). SPE allows the convenient differential fractionation of phytohormones with distinct

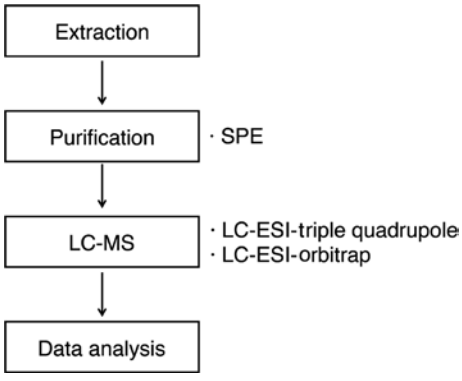

Figure 4: General workflow of liquid chromatography-mass spectrometry (LC–MS) analysis.
SPE, solid-phase extraction; ESI, electrospray ionization.

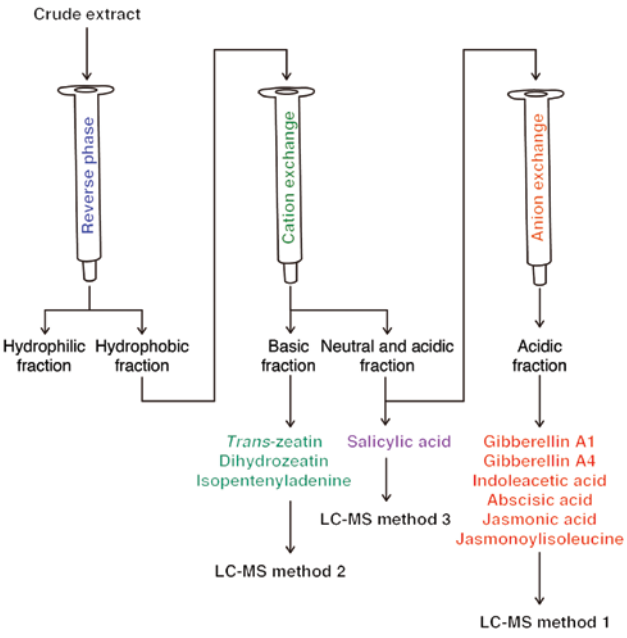

Figure 5: Example of solid-phase extraction of phytohormones for simultaneous analysis.
The scheme represents the procedure of purification and fractionation of phytohormones reported in Tsukahara et al. (2015) and Mikami et al. (2016). Ten hormones were fractionated into three fractions. The basic fraction selectively eluted from the cation exchange cartridge includes *trans*-zeatin, dihydrozeatin, and isopentenyladenine. This fraction was analyzed with LC–MS method 2. Salicylic acid was set aside after the elution of neutral and acidic fraction from the cation exchange cartridge, since its retention time overlaps with gibberellin A_1 and indoleacetic acid, and was analyzed with LC–MS method 3. The remaining hormones were eluted from the anion exchange cartridge as the acidic fraction and successively analyzed by LC–MS method 1. For the detailed procedure of fractionation and LC–MS methods, see Tsukahara et al. (2015) and Mikami et al. (2016).

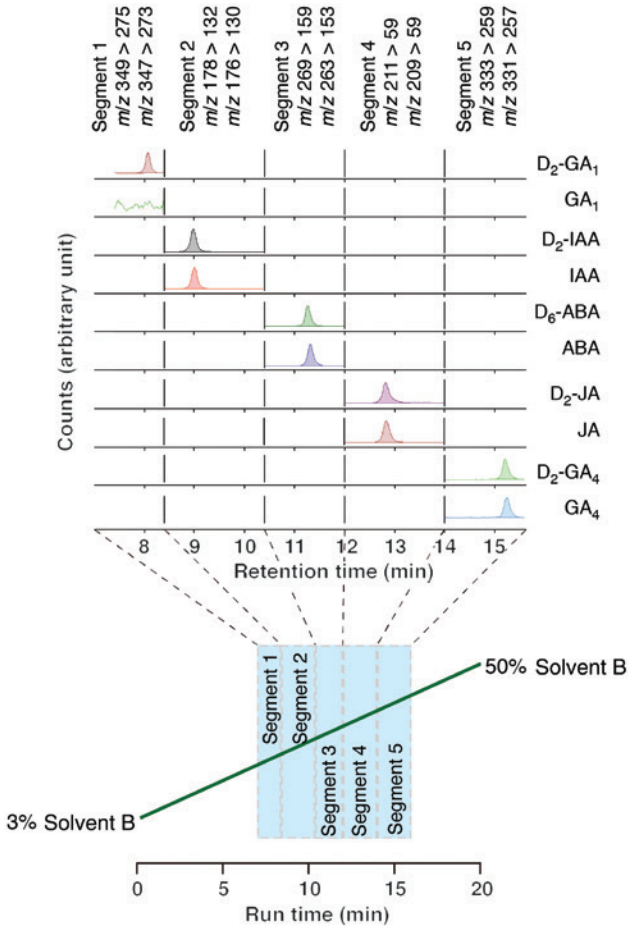

Figure 6: Sequential analysis of multiple phytohormones by a single LC–MS run.
An example of sequential analysis of five hormones in an acidic fraction in a single run using the LC–MS method 1. Phytohormones were extracted from flower buds (~20 mg) of *Arabidopsis thaliana* by the method described in Tsukahara et al. (2015). The single run, using the LC–MS method 1 with a gradient of solvent, was divided into more than five segments. Configuration of quadrupole mass filters of each segment was set for *m/z* of each pair of endogenous hormone and internal standard (Segment 1, gibberellin A_1 [GA_1]; Segment 2, indoleacetic acid [IAA]; Segment 3, abscisic acid [ABA]; Segment 4, jasmonic acid [JA]; Segment 5, gibberellin A_4 [GA_4]). The setup of the quadrupole mass filter was changed every 0.2 s to acquire signals between the endogenous hormone and the internal standard in a given segment. Green slope indicates gradient protocol of liquid chromatography (LC) in which the concentration of solvent B increased from 3% to 50% in 20 min.

chemical structure/characteristics. A well-designed SPE procedure can effectively reduce the matrix effect.

Simultaneous analysis of multiple phytohormones

Together with the development of appropriate pretreatment methods using SPE as discussed above, LC–MS now enables the simultaneous analysis of multiple phytohormones without the need to prepare samples for each hormone (e.g. Tokuda et al. 2013). A sufficient pretreatment procedure permits analysis with small amounts of plant tissue or thallus (~20–100 mg dry weight) (Mikami et al. 2016). Therefore, it is not difficult to increase the number of samples to acquire much information. Furthermore, variation in the data set is minimized, and hormones can be compared with high precision because they come from the same sample.

Analyses of multiple phytohormones often exhibit unexpected results. For example, Tsukahara et al. (2015) initially hypothesized that differences in auxin content were involved in the observed yield differences between two rice (*Oryza sativa* L.) cultivars exposed to ozone.

However, they found that JA was involved in the ozone response instead of IAA. Moreover, their working hypothesis was that differences in the contents of auxin and cytokinins in the calli of barley explain the difference in regeneration efficiency among cultivars in the course of experiments reported by Hisano et al. (2016) and Rikiishi et al. (2015). However, these two independent studies demonstrated that regeneration capacity could be explained by differences in ABA content. These examples suggest that the effectiveness of the simultaneous analysis of multiple phytohormones is evident.

The latest technical advances in phytohormone analysis have focused on developing less labor-intensive and comprehensive analyses rather than improving sensitivity and accuracy for a specific hormone. In a study of rice phytohormones, utilizing four SPE columns sequentially to fractionate phytohormones from a sample and analyzing by UPLC(R)-MS/MS, the contents of up to 40 phytohormones were determined simultaneously (for details, see Kojima et al. 2009). Hormones present in relatively high amounts, such as IAA, ABA, and JA (Table 1), can be measured with a less labor-intensive pretreatment and with sufficient precision, but those present in relatively low amounts, such as GAs and cytokinins, are difficult to detect without such pretreatment. It is feasible to determine their contents even under suboptimal conditions. Quantification of strigolactones and brassinosteroids appears to be difficult without more labor-intensive pretreatment SPE procedures, GC-MS combined with high-resolution HPLC, or GC-MS combined with the quadruple/time-of-flight mass spectrometry (Umehara et al. 2008, Ding et al. 2013). The optimal pretreatment procedure for basic cytokinins is different from that used for acidic phytohormones. Therefore, the recovery rate of cytokinins is improved if they are purified separately from acidic hormones: there is a trade-off between sensitivity of analysis and simplicity of pretreatment. Analysis methods can be variable based on the purpose of the study.

Many phytohormones form interaction networks, and ultimately control the development and environmental responses of terrestrial plants in a coordinated way (Jaillais and Chory 2010). The biosynthetic pathways of many angiosperm phytohormones have recently been elucidated, except for that of auxin, which is still under debate (Zhao 2014, Kamiya 2010, Mano and Nemoto 2012, Tivendale et al. 2014). The simultaneous analysis of multiple phytohormones using LC–MS/MS is a powerful tool to understand the profile of phytohormones, especially in lower terrestrial plants and seaweeds for which the existence and mechanisms of phytohormone biosynthetic pathways are not well understood.

Practical aspects of quantifying phytohormones in red seaweeds

Optimization of extraction methods

Seaweed thalli have peculiar physical and chemical properties with variable morphology, sometimes filamentous, sometimes fan shaped, delicate, or even calcified, which require different approaches for the extraction procedure. Red seaweed thalli usually contain viscous polysaccharides in the cell walls, such as agar and carrageenan, as well as many unidentified secondary metabolites. Accordingly, it may be necessary to optimize procedures for extracting hormones from seaweeds. Mikami et al. (2016) extracted hormones from lyophilized *Pyropia yezoensis* gametophytes with a solvent containing 1% acetic acid, 19% water, and 80% acetonitrile. The viscosity of the extraction solvent increased during grinding of the sample in a mortar and the debris of algal material became stuck to the bottom of the mortar, so that it was difficult to transfer the sample to a centrifuge tube for subsequent experimental steps. After incubation for 1 h at 4°C, the solvent became less viscous and transfer was possible (unmentioned details in Mikami et al. 2016). Extracts from the gametophytes of another red seaweed, *Bangia fuscopurpurea*, also became very viscous, but those of *P. yezoensis* sporophytes did not. Such viscosity is not normally seen with samples of terrestrial plants. Extracts from the brown seaweeds *Ectocarpus siliculosus*, *Saccharina japonica*, *Alaria crassifolia*, and *Scytosiphon lomentaria* did not become viscous in the same extraction procedure (unmentioned details in Mikami et al. 2016). This may be because of the unique polysaccharide composition of red seaweeds.

One may question whether such a viscous solvent would affect the extractability of hormones. Different solvents were used sequentially to extract hormones in the following order: 80% acetonitrile containing 1% acetic acid and 19% water, 80% methanol containing 1% acetic acid and 19% water, 80% acetone containing 1% acetic acid and 19% water, and chloroform:methanol (1:1) containing 1% acetic acid. As reported previously, IAA was extracted efficiently by the first extraction step (~95%, Table 2; Mikami et al. 2016). The extraction efficiency of ABA and iP was also high at the first step (~95%, Table 2, Figure 7; Mikami et al., 2016); however, SA was not efficiently extracted by the complete series of extraction steps (Table 2). This suggests that although LC–MS analysis confirmed the existence of SA in *P. yezoensis* gametophytes, the result of the quantitative analysis is not satisfactory.

Table 2: Recovery rate of phytohormones from gametophytes of the red alga *Pyropia yezoensis* using a series of solvents.

Extraction step	1st (MeCN[1])	2nd (MeOH[2])	3rd (acetone)	4th (Chl/Met[3])
IAA	95.3	3.6	0.7	0.3
iP	97.7	2.3	0.0	0.0
ABA	95.0	5.0	0.0	0.0
SA	47.5	17.3	9.1	26.1

Recovery rate (%) = counts(i)/$\Sigma^{4}_{i=1}$ counts(i) ×100. Here, i indicates each extraction step. [1]acetonitrile, [2]methanol, [3]chloroform/methanol (1:1). n = 2. Data are modified from Mikami et al. (2016).

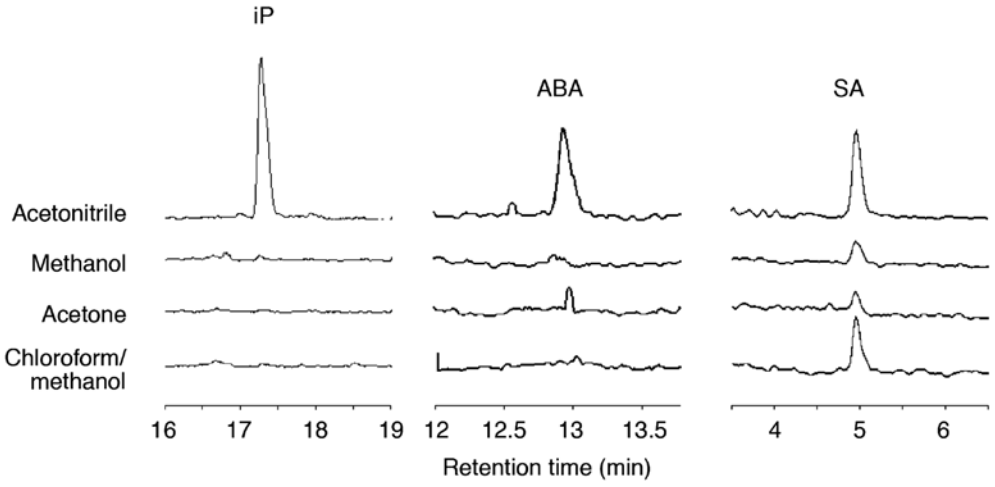

Figure 7: Difference in extractability of isopentenyladenine (iP), abscisic acid (ABA), and salicylic acid (SA) from gametophytes of the red alga *Pyropia yezoensis*.
Phytohormones were extracted sequentially with four different solvents as presented in Table 2. The data are modified from Mikami et al. (2016). Peaks corresponding to iP and ABA were barely detected beyond the first extraction step, indicating that the solvent (consisting of 80% acetonitrile and 1% acetic acid) extracted these hormones efficiently. SA appeared in all four tested extraction steps, indicating that extraction of SA from *P. yezoensis* was inefficient using the first three solvents.

To precisely quantify SA content in *P. yezoensis*, an alternative extraction procedure, specific to SA, should be developed alongside a simultaneous extraction of multiple hormones. The extractability of hormones is not greatly affected by the extract characteristics in general, but it should be checked routinely depending on the algal species used.

Despite the presence of polysaccharides distinct from those in terrestrial plants, SPE extraction from red seaweeds and subsequent MS analysis (Mikami et al., 2016) was similar in efficiency to those reported for terrestrial plants (wheat, Iehisa et al. 2013, 2014; rice, Tsukahara et al. 2015; barley, Rikiishi et al. 2015 and Hisano et al. 2016; *Arabidopsis*, Lu et al. 2015 and Takagi et al. 2016). In the course of that work (Mikami et al., 2016), phytohormones were extracted from both lyophilized and fresh seaweed thalli after freezing in liquid nitrogen, which made the fresh samples easier to grind. Another difficulty sometimes encountered in the extraction process, besides the transient viscosity, was that when the fronds of brown seaweeds were ground in the solvent, the solvent sometimes separated into two phases. This did not happen with extracts from terrestrial plants or red seaweeds. Figure 8 shows a typical appearance of extracts of rice leaf blades and fronds of the brown seaweeds *Scytosiphon lomentaria*, *Saccharina japonica*, and *A. crassifolia*, which were ground and suspended in 80% acetonitrile containing 1% acetic acid according to the method reported by Mikami et al. (2016). Powdery tissues of brown seaweeds (but not rice) formed an aggregate that lasted more than 30 min and dispersed after a 1-h incubation at 4°C. After clearing by centrifugation, the supernatant separated into two phases: the upper phase contained chlorophyll and the lower phase was colorless. In general, the volume of lower phase was smaller than the upper phase. The lower phase of *Saccharina japonica* was larger than that for the other two brown algae, *Scytosiphon lomentaria* and *A. crassifolia* (Figure 8). In a preliminary experiment, there was no

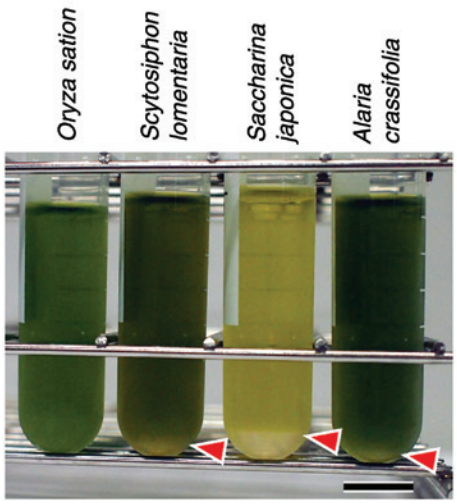

Figure 8: Problems with phase separation in the extraction of phytohormones from brown seaweeds.
Rice (0.3 g fresh weight) and the fronds of three species of brown seaweed (0.1 g dry weight) were ground in liquid nitrogen with a mortar and a pestle, followed by extraction of phytohormones with 4 ml of 80% acetonitrile containing 1% acetic acid. Supernatant is shown after centrifugation at 3000 *g* for 10 min at 4°C. Transparent lower phase is indicated by an arrowhead. Detail of methods as described by Mikami et al. (2016).

apparent interference in the detection of phytohormones by LC–MS (unmentioned details in Mikami et al. 2016). However, a careful investigation of the extraction rate of hormones should be conducted in the future to quantify hormones in brown seaweeds.

This may not be a critical problem for using SPE for algal samples because it was reported in previous papers conducting simultaneous multihormone analysis of red seaweeds (Wang et al. 2014b, Mikami et al. 2016).

Investigation of internal standards

There are several ways to reduce matrix effects when quantifying compounds by LC–MS. The matrix effect is more or less unavoidable when quantifying phytohormones using LC-ESI-MS, as discussed above. Methods utilizing internal standards labeled with stable isotopes, such as deuterium or ^{13}C, are often employed to mitigate or avoid matrix effects (Wieling 2002). Because such standards have almost equal physicochemical properties except for molecular mass, it can be assumed that the extent of ion suppression is also equal to that of endogenous hormones and internal standards. Therefore, phytohormone contents are normalized to the intensity of the spiked internal standards.

Standard addition is another way to resolve matrix effect (Gergov et al. 2015). In this method, one estimates the concentration of the target compound by adding standard reference materials (not labeled internal standards) at a known concentration and examining the y-intercept of the measured working curve. This does not require costly isotope-labeled standards, but it requires multiple runs of LC-MS to generate a working curve. The addition of the standard to the pretreated sample does not take into account the recovery rate during pretreatment, but only the matrix effect.

A state-of-the-art analysis column is able to distinguish the difference in retention time (RT) even between endogenous phytohormones and deuterium-labeled internal standards (Turowski et al. 2003); for example, RT = 9.87 min for IAA and RT = 9.73 min for D_7-IAA ($^2H_7$-IAA, for the structure see Figure 9), a difference of 0.14 min (unpublished data examined as in Mikami et al. 2016). This difference in RT does not occur for ^{13}C-labeled internal standards. Nevertheless, occasionally mass chromatogram peaks may not be separable due to interference from

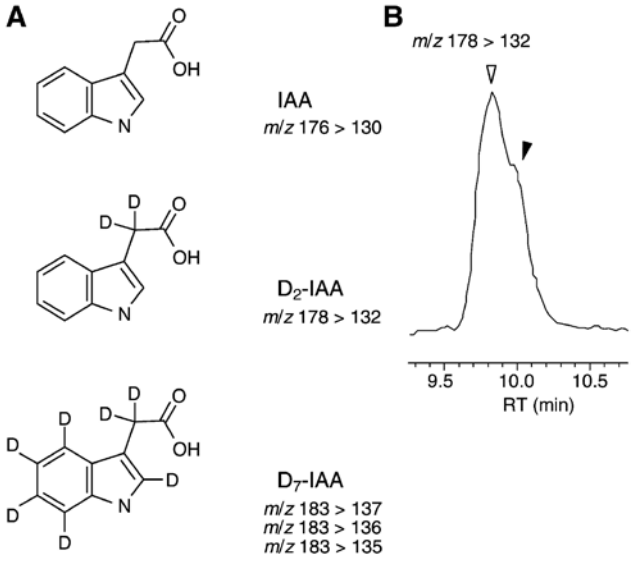

Figure 9: Structure of indoleacetic acid (IAA) and its deuterium-labeled derivatives (D_2-IAA, D_7-IAA), and mass chromatogram at mass-to-charge ratio (*m/z*) 178 > 132.
(A) Hydrogen atoms replaced by deuterium are shown as D. *m/z* of the precursor ion and fragment ion of IAA are 176 and 130, respectively; *m/z* of the precursor and fragment ions of D_2-IAA are 178 and 132, respectively. D_7-IAA yields three different fragment ions, *m/z* 135, 136, and 137 from the precursor ion, *m/z* 183. (B) Mass chromatogram of extracts of *Pyropia yezoensis* at *m/z* 178 > 132. Results for D_2-IAA and a contaminant overlap: open arrowhead indicates the mass chromatogram peak corresponding to D_2-IAA, and closed arrowhead indicates a shoulder corresponding to the contaminant possessing the same multiple reaction monitoring transition, but slightly delayed retention time (RT). Data are modified from Mikami et al. (2016).

a substance whose MRM peak overlaps with that of phytohormones. To judge whether the chromatogram peak was due to a phytohormone or a contaminant, co-injection of the standard reference material is helpful. If the chromatogram contains a contaminant, a shoulder peak may appear or the shape of the peak may be altered. In addition to endogenous phytohormones, sometimes the peak of contaminant may overlap with that of an isotope-labeled internal standard. The occurrence of interference with the MRM transition of the internal standard is reported in a previous study (Mikami et al. 2016). To distinguish the internal standard from contaminants, it is necessary to prepare two extracts, one with the internal standard added, and another without the internal standard, and to analyze with LC–MS. If an overlapping contaminant is present, a peak is observed even in the extracts without the internal standard added. If a peak with a "shoulder" or a "split" is observed, the sample may be contaminated with an overlapping peak; a blurred column and excess injection volume are also possibilities. During analysis of IAA in *Pyropia yezoensis*, a shoulder peak was found in a chromatogram of *m/z* 178 > 132. This corresponded with D_2-IAA ($^2H_2$-IAA) (Figure 9; Mikami et al. 2016) and was shown to be a contaminant. The problem was solved by using an alternative standard, D_7-IAA (for detail, see Mikami et al. 2016).

A satisfactory pretreatment procedure successfully reduced problems of ion suppression effect in the analysis of IAA, iP (Figure 1F), tZ (*trans*-zeatin) (Figure 1G), ABA, SA and GA_1 (Figure 1H), GA_4 (Figure 1I), dihydrozeatin (Figure 1J), JA, and jasmonoylisoleucine (Figure 1K) in leafy gametophytes and filamentous sporophytes of *P. yezoensis* and *Bangia fuscopurpurea* (Mikami et al. 2016).

Phytohormones in red seaweeds: presence of particular hormones and possible biosynthetic pathways

Improved chemical analysis unambiguously detected IAA, ABA, iP, and SA in thalli of the Bangiophycean algae *Pyropia yezoensis, Pyropia haitanensis*, and *Bangia fuscopurpurea* (Wang et al. 2014b, Mikami et al. 2016). Neither study was able to detect jasmonic acid (JA) or gibberellins (GAs) at the detection limits of their methods.

In addition to known phytohormones, the functional significance of precursors of JA and GA has recently been demonstrated in nonvascular terrestrial plants like mosses and liverworts. It is well known that (9*S*,13*S*)-12-oxo-phytodienoic acid (*cis*-(+)-OPDA; hereafter OPDA), a precursor of JA, is biologically active and involved in the regulation of growth and defense in the moss *Physcomitrella patens* and the liverwort *Marchantia polymorpha* (Stumpe et al. 2010, Ponce De León et al. 2012, Yamamoto et al. 2015). However, *P. yezoensis* and *B. fuscopurpurea* have no OPDA (Mori, Matsuura and Mikami, unpublished), which is consistent with the lack of JA in these Bangiophyceae. Moreover, in *P. patens*, despite the absence of bioactive GA_1 and GA_4, the precursors *ent*-kaurene and *ent*-kaurenoic acid and also their metabolites other than GAs regulate red light-dependent cellular differentiation and blue light-dependent negative phototropism (Hayashi et al. 2010, Miyazaki et al. 2014, 2015). However, little is known so far about the presence of these molecules in red seaweeds, and the confirmation of the presence of GA precursors in red seaweeds is needed to understand the evolutional diversity of GA biosynthesis in plants.

Biosynthetic pathways of phytohormones in algae have hardly been studied yet, and in red seaweeds, it is not realistic to demonstrate biosynthetic pathways of phytohormones by genetic approaches. On the other hand, it is conceivable that recently emerging algal genomic information can be compared with known genomic information of terrestrial plants. It is indisputable that genomic analysis is a critical tool in answering these questions.

Genome-wide analysis of phytohormone biosynthesis and signaling genes

Genome information for Bangiophycean algae

The first genomic analysis of a seaweed was performed for the brown alga *Ectocarpus siliculosus* (Cock et al. 2010). In this organism, auxin functions in the regulation of sporophyte branching patterns. Established genomic data were used to search for factors involved in the biosynthesis and signal transduction of auxin (Le Bail et al. 2010).

In contrast to brown algae, little is known about the physiological functions of hormones in red seaweeds, or the molecular basis of metabolism and cell signaling. To tackle this problem, a powerful approach would be to search for the presence of homologues of known phytohormone-related genes in red seaweeds. To date, genomic analysis of red seaweeds has only been performed in the Bangiophyte *Pyropia yezoensis* (Nakamura et al. 2013) and

the Florideophyte *Chondrus crispus* (Collén et al. 2013). Although information about the *P. yezoensis* genome is accessible online (http://nrifs.fra.affrc.go.jp/ResearchCenter/5_AG/genomes/nori/index.html), there is, as yet, no such website for the *C. crispus* genome. In addition to these genomic analyses, abundant expression sequence tag (EST) information is available for the Bangiophycean algae *Porphyra umbilicalis* and *Porphyra purpurea* (Chan et al. 2012a,b, Stiller et al. 2012), and the *Nori*BLAST website (http://dbdata.rutgers.edu/nori/) has been established. Databases to search for phytohormone-related genes are now available for *P. yezoensis*, *P. umbilicalis*, and *P. purpurea*. These genomic information resources for Bangiophycean algae can provide supportive evidence of functional metabolic pathways.

Genome-wide survey for genes involved in phytohormone biosynthesis

As described above, IAA, iP, ABA, and SA, but not GAs and JAs, have been identified in red seaweeds using improved analytical methods (Mikami et al. 2016). If algae produce phytohormones, it is conceivable that genes encoding biosynthetic enzymes of the phytohormones are found in the genomic sequence. To scrutinize these analytical results for molecular biological information, it is necessary to conduct a bioinformatic search of homologous genes that are involved in the biosynthetic pathways and signal transduction systems of plant hormones in red seaweeds. An extensive genome-wide survey was performed for genes involved in the biosynthesis of phytohormones such as IAA, iP, ABA, and SA, in the Bangiophycean seaweeds *Pyropia yezoensis*, *Porphyra umbilicalis*, and *Porphyra purpurea* (Mikami et al. 2016).

Cytokinins, iP, tZ, and DHZ are synthesized from dimethylallyl diphosphate (DMAPP) in the methylerythritol phosphate (MEP) pathway, whereas DMAPP derived from the mevalonate (MVA) pathway is catalyzed to *cis*-zeatin (cZ) (Sakakibara 2006). The MEP pathway-derived DMAPP is converted to an iP nucleotide with adenine phosphate-isopentenyltransferase (IPT) and then to a tZ nucleotide with CYP735A. This is followed by the conversion of iP and tZ nucleotides to cytokinins with cytokinin riboside 5′-monophosphate phosphoribohydrolase (LONELY GUY, LOG) as the final step of cytokinin biosynthesis (Sakakibara 2006). ESTs in both *P. purpurea* and *P. umbilicalis* were found encoding a homologue of LOG (Mikami et al. 2016); however, neither CYP735A nor IPT homologues were identified (Table 3). Although these findings indicate the presence of a branch of the cytokinin biosynthetic pathway in these seaweeds, it is possible that iP is biosynthesized *in vivo* as the vital cytokinin in Bangiophyceae.

Despite some ambiguity about the presence of the ABA precursors violaxanthin and neoxanthin in red seaweeds (reviewed in Mikami and Hosokawa 2013), ABA has been detected in several red seaweed species (Yokoya et al. 2010, Wang et al. 2014b, Mikami et al. 2016). By comparative genome survey, genes encoding zeaxanthin epimerase (ZEP) – predicted to catalyze the production of violaxanthin from zeaxanthin – were identified in *P. yezoensis* as well as in *P. purpurea* and *P. umbilicalis* (Mikami et al. 2016). In addition, genes encoding 9-*cis*-epoxycarotenoid dioxygenase (NCED), xanthoxin dehydrogenase (XanDH, also known as ABA2), and abscisic aldehyde oxidase (AAO3) involved in ABA biosynthesis from carotenoids (Nambara and Marion-Poll 2005) were also identified in Bangiophycean seaweeds (Mikami et al. 2016), although biochemical validation of the enzymatic activity is needed. These findings strongly support the presence of an ABA biosynthetic pathway in Bangiophyceae, which is consistent with a proposal of an ancient evolutionary origin of the ABA biosynthetic pathway (Takezawa et al. 2011).

No homologues of IAA or SA biosynthetic genes were found in Bangiophyceae as shown in Table 3. This contrasts with the brown alga *Ectocarpus siliculosus*, in which the presence of several tryptophan-dependent auxin biosynthetic genes, such as CYP79B, CYP71A, AMI1, and nitrilase (see Table 1), in the genome was proposed (Le Bail et al. 2010). There are two possibilities: (1) red seaweeds have phytohormone biosynthetic pathways different from those of terrestrial plants, or (2) red seaweeds do not biosynthesize IAA and SA endogenously but obtain these hormones from neighboring microorganisms.

Genome-wide survey for genes involved in signal transduction of phytohormones

A genomic survey of genes in *Pyropia yezoensis*, *Porphyra umbilicalis*, and *Porphyra purpurea* that are involved signal transduction of phytohormones indicated that genes encoding proteins similar to known factors involved in the signal transduction of IAA, iP, ABA, and SA were absent (see Table 3). Remarkably, there were no homologues of genes encoding phytohormone receptors, including the auxin receptor TIR1 (Dharmasiri et al. 2005); the cytokinin receptors AHK2, AHK3, and CRE1/AHK4 kinases (Inoue et al. 2001, Nishimura et al. 2004); the PYR/PYL/RCAR ABA receptor proteins (Cutler et al.

Table 3: Plant hormone biosynthesis and signaling genes absent in three red seaweeds, *Pyropia yezoensis*, *Porphyra umbilicalis*, and *Porphyra purpurea*.

Gene	Gene product
Auxin biosynthesis	
TAA1	Tryptophan aminotransferase
TAR1	Tryptophan aminotransferase
TIR2	Tryptophan aminotransferase
TDC	Tryptophan decarboxylase
CYP79B2/B3[a]	Trp-specific cytochrome P450 monooxygenase
CYP71A13[a]	Indoleacetaldoxime dehydratase
iaaM	Flavin containing amine oxidoreductase
AMI1	Indole-3-acetamide hydrolase
iaaH	Indole-3-acetamide hydrolase
YUC (YUCCA)	Flavin monooxygenase-like protein
IPDC	Indole-3-pyruvate decarboxylase
AO	Aldehyde oxidase
NIT	Nitrilase
Auxin signaling	
TIR1	Transporter inhibitor response 1
ABP1	Auxin-binding protein 1
IBR5	Indole-3-butylic acid response 5
Aux/IAA	Auxin-responsive protein
ARF	Auxin response factor
Auxin transport	
PIN	Auxin efflux carrier component (Pin-formed)
AUX1	Auxin influx carrier protein 1
AXR4	Auxin response 4
Cytokinin biosynthesis	
IPT	Adenylate isopentenyltransferase (Cytokinin riboside 5′-monophosphate)
YCP735A[a]	Cytochrome P450 monooxygenase
Cytokinin signaling	
CHASE-domain HK	*Arabidopsis* histidine kinase 4 (AtHK4) [cytokinin response 1 (AtCRE1), cytokinin receptor (CRE1), phosphoprotein phosphatase 4 (AHK4)], *Arabidopsis* histidine kinase 3 (AtHK3) [phosphoprotein phosphatase 3 (AHK3)], *Arabidopsis* histidine kinase 2 (AtHK2) [phosphoprotein phosphatase 2 (AHK2)],
HP	Histidine-containing phosphotransfer protein
RR	Response regulator
ABA signaling	
PYR/PYL/RCAR	Abscisic acid receptor (PYR), PYR1-like protein (PYL), regulatory components of ABA receptor (RCAR)
PP2C56[a]	Protein phosphatase 2C 56 (Abscisic acid-insensitive 1; ABI1)
SnRK2[a]	SNF1-related kinase 2
ABI5	Abscisic acid-insensitive 5 (bZIP transcription factor)
ABF	Abscisic acid responsive elements-binding factor (bZIP transcription factor)
SA biosynthesis	
ICS	Isochorismate synthase
IPL	Isochorismate pyruvate lyase
PAL	Phenylalanine ammonia lyase
BA2H	Benzoic acid 2-hydroxylase
CM	Chorismate mutase
SA signaling	
NPR1	Nonexpressor of pathogenesis-related gene 1
NPR3	Nonexpressor of pathogenesis-related gene 3
NPR4	Nonexpressor of pathogenesis-related gene 4
TGA2,3,7	bZIP transcription factor
SSN2	Suppressor of RNA polymerase B

[a]Genes whose correct counterparts were not determined because of the presence of many homologous genes.

2010, Klingler et al. 2010); or NPR proteins, receptors for SA (Wu et al. 2012). Moreover, homologues of signal transducers and transcription factors known to participate in phytohormone-inducible gene expression were not found; histidine-transfer proteins and response regulators involved in cytokinin signaling (To and Kieber 2008), e.g. IBR5 (Monroe-Augustus et al. 2003), Aux/IAA (Abel et al. 1994, 1995), and ARF (Ulmasov et al. 1997, Guilfoyle et al. 1998), which participate in auxin signaling, are also missing, and no homologues of the auxin transporters PIN (Okada et al. 1991, Gälweiler et al. 1998) or AUX1 (Bennett et al. 1996, Marchant et al. 1999) were present. These findings strongly suggest that the modes of action of phytohormones in Bangiophycean seaweeds differ from those of phytohormones in plants, and that the evolution of signal transduction pathways in red algae has diverged from that in plants.

The absence of homologues of genes encoding auxin signal transduction components has also been reported in the brown seaweed *Ectocarpus siliculosus* (Le Bail et al. 2010), the unicellular red alga *Cyanidioschyzon merolae*, and the green algae *Chlamydomonas reinhardtii* and *Volvox carteri* (Lau et al. 2009, De Smet et al. 2011). Thus, algae appear to have molecular mechanisms for the perception and intracellular signaling of auxin different from those elucidated in terrestrial plants.

Are phytohormones synthesized by and transferred from symbiotic microorganisms?

It is clear that seaweeds largely lack known factors involved in the biosynthesis of auxin and SA (Table 3). Thus, the origin of IAA and SA in Bangiophyceae is unresolved. To date, the origin of the auxin biosynthetic pathway in land plants is controversial; for instance, Wang et al. (2014a, 2016) proposed that auxin biosynthesis originated in charophytes, although Turnaev et al. (2015) refuted this hypothesis. However, these studies both suggest that the auxin biosynthetic pathway is absent in red algae. Phylogenetic analyses might reveal that auxin biosynthesis developed through bacterial interactions with land plants (Yue et al. 2014). Therefore, the most plausible suggestion involves epiphytic microorganisms that are found on the surfaces of seaweeds. Based on the most extensive studies of interactions between bacteria and marine green seaweeds to date (Joint et al. 2007, Wichard 2015), the association of seaweeds with epiphytic bacteria is commonly thought to be essential for their normal growth, morphogenesis, and reproduction (Egan et al. 2013, Singh and Reddy 2014, Wichard 2015, Liu et al. 2017). In addition to exchanges of nutrient chemicals and morphological regulators (Egan et al. 2013, Singh and Reddy 2014, Wichard 2015), bacterium-derived IAA is involved in bud formation in the Florideophyte *Gracilaria dura* (Singh et al. 2011a). With regard to phytohormones, it was recently reported that diatoms receive IAA from their symbiotic bacteria, and these are required for efficient growth (Amin et al. 2015). In addition, a few reports have shown that epiphytic microorganisms transfer active growth regulating compounds to macroalgae (Spoerner et al. 2012, Grueneberg et al. 2016). To demonstrate that phytohormones can be supplied to red seaweeds through seawater, axenic cultures of algae are essential. However, such cultures of macroalgae are not available, apart from a few exceptions (Fries 1975, Singh et al. 2011b, Spoerner et al. 2012, Grueneberg et al. 2016). At least two pathways of IAA biosynthesis are suggested in bacteria (Patten and Glick 2002). One is tryptophan dependent (Manulis et al. 1998, Carreno-Lopez et al. 2000), and another is tryptophan independent (Prinsen et al. 1993). A complete understanding of IAA synthesis in seaweeds may require a meta genomic analysis of the bacterial flora attached to seaweed thalli.

Taken together with the presence of SA-biosynthesizing bacteria (De Meyer and Höfte 1997, Press et al. 1997), we hypothesize that epiphytic bacteria contribute to the supply of IAA and/or SA to Bangiophyceae. Identifying phytohormone-producing bacteria will therefore be important to establish the origin of IAA and SA in Bangiophycean seaweeds.

Acknowledgments: The authors are grateful for support received from the Joint Research Program at the Institute of Plant Science and Resources, Okayama University, and the Japan Advanced Plant Science Network. This work was supported in part by a KAKENHI grant-in-aid for the support of scientific research (no. 15H0453905) and the Ohara Foundation for Agricultural Research.

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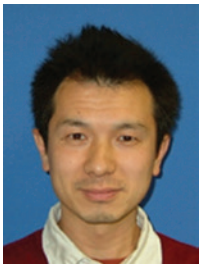
Bionotes



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