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Comparison of proteomic profiles of the phaeophyte *Saccharina japonica* thalli proximal to and beneath the front of epiphytic hydrozoan colonies against healthy tissue

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Abstract: The stoloniferous hydrozoan *Obelia geniculata* commonly colonizes macroalgae such as *Saccharina japonica*. Each *Obelia* colony consists of thread-like hydrorhizae attached to the seaweed thallus. The early signaling proteins of epiphytic contamination can be identified using proteomics. To isolate these early signals, parts of the thallus proximal to the hydrozoans were separated from beneath the colony front and from healthy tissue. From the proteomic profiles of *S. japonica*, we detected 110 protein spots from tissue proximal to hydrozoan colonies (56 increased, 53 decreased, and 1 unchanged in expression relative to healthy tissue) and 133 spots from tissue at the colony front (67 increased, 60 decreased, and 6 unchanged in expression). Of the proteins with increased and decreased expression, SIPA1L1 and actin increased strongly only in the proximal tissues, while NEK2 kinase decreased. CIPK20 and SIPA1L2 increased markedly in both the colony-front and proximal tissues, while CaMK2N2 and GK25369 decreased in both tissues. ATPase β , ADA, kinesin, and HECT domain proteins increased only in the colony-front tissues. Among them, SIPA1L2 increased strongly in both the thallus tissues proximal to the hydrozoans and those beneath the colony front, but was not expressed in response to bryozoan infection.

Keywords: biomarker; hydrozoan; MALDI-TOF/MS; proteomics; *Saccharina japonica*.

Introduction

The edible brown seaweed *Saccharina japonica* has been extensively cultivated in East Asia. In 2016, 397,852 tons

(wet weight) of *S. japonica* were produced by farming; an additional 11 tons (wet weight) were harvested from natural populations in Korea (Korea Fisheries Association 2017). The seaweed thalli are consumed as popular health seafood and used as abalone feed. Epiphytism of brown algal thalli is a major problem in seaweed aquaculture, as it reduces product quality and yield. Hydroids, bryozoans, amphipods, copepods, and gastropods are some of the most abundant epiphytes on the blades of seaweeds (Peteiro and Freire 2013). Unsightly, coarse colonizers of seaweed thalli also markedly reduce the commercial value of the thalli. One of the most common epiphytic colonizers on *S. japonica* thalli is the stoloniferous hydrozoan *Obelia geniculata* Linnaeus. Its planktotrophic larvae settle on the kelp thalli and give rise to extensive colonies covering the surface of the seaweed. The colony produces horizontal thread-like roots. Over the last two decades, there have been severe outbreaks, especially on late-harvested *S. japonica* (Park and Hwang 2012). Infestation is characterized by the appearance of hairy colonies on thalli. Hydrozoans contain high amounts of zinc at levels higher than the recommended range in food (Getachew et al. 2015a). As evaluated for food, hydrozoans and/or hydrozoan-colonized tissues seriously reduce the quality of the seaweed so that the epiphytic hydrozoans must be removed from the seaweed thallus prior to its use. Most studies thus far have focused on the biology of the hydrozoans. The hydrozoan *O. geniculata* is a sessile epiphyte distributed throughout the world (Cornelius 1990). Hydroid colonies on *S. japonica* thalli reduced seaweed tissue viability by 77% compared with healthy tissues (Getachew et al. 2015a).

Upon colonization, the host seaweed can respond physiologically, which may lead to changes in life activities at biochemical and molecular levels in the thalli. Although the biochemical mechanism is unclear, following removal of the hydrozoans, *Saccharina japonica* tissues beneath the colonies exhibit elevated selenium, iodine, and docosahexaenoic acid levels and reduced copper, cadmium, nickel, chromium, and erucic acid levels compared with the distal healthy thallus tissue (Getachew et al. 2015a). When hydrozoans colonize kelp thalli, the hydrozoans also affect protein expression of the host by inducing signal transduction in the host seaweed. We previously found that 7 and 16 proteins were significantly up-regulated and

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down-regulated, respectively, in hydrozoan-colonized tissues (Getachew et al. 2016). Therefore, we postulated that the thallus tissue proximal to hydrozoan colonies and tissue at the colony front may be directly or indirectly influenced by epiphyte secretion, similar to the ways in which self-recognition affects plant communication and defense (Karban and Shiojiri 2009). The molecular impacts and up- and down-expressed proteins in the colony-front and proximal tissues could possibly be used as biomarkers in future studies for early diagnosis and improving strain resistance to epiphytic infestation.

In this paper, early hydrozoan colonization marker proteins from colony-front and proximal tissue sections of colonized *Saccharina japonica* thalli were identified and their primary roles in cellular activities are discussed.

Materials and methods

Seaweed, hydrozoans, and reagents

Fresh thalli of late harvested *Saccharina japonica* (J.E. Areschoug) C.E. Lane, C. Mayes, Druehl et G.W. Saunders were collected from Gijang aquaculture farm, Busan, Korea in June 2016 and 2017. A voucher specimen was deposited in the author's laboratory (Y.K. Hong). The seaweed tissues were acclimatized in an aquarium tank for 3 days (Getachew et al. 2018). For detection of epiphyte infection, namely induced defense responses from neighboring tissues, different tissue sections of the thalli covered by the hydrozoan *Obelia geniculata* colony were selected (Figure 1), and colonies were gently scraped off with a stiff plastic sheet. Thallus tissue proximal to the hydrozoan colony (the 1-cm zone outside the boundary of the colony) and tissue from the colony front (the 1-cm zone under the newly formed front of the colony after removing the hydrozoans) were immediately freeze dried, ground to a fine powder, and kept at -70°C before analysis. Distal healthy thallus tissue located at least 30 cm from the colony was treated in the same way and used as a control. The reagents used in this study were of analytical grade from Sigma-Aldrich Co., St. Louis, MO, USA unless otherwise stated.

Protein electrophoresis and quantification

Protein was prepared by the method of Getachew et al. (2014). Briefly, the seaweed powder [0.5 g in 5 ml lysis

Figure 1: *Saccharina japonica* thallus sections defined as thallus tissue proximal to hydrozoan colonies and tissue at the colony front. The proximal tissues were collected from the 1-cm zone outside the boundary of the colony. The colony-front tissues were obtained from the 1-cm zone under the newly formed front of the colony after removing the hydrozoans. Inset photo shows hydrozoans on the brown alga *S. japonica*.

solution (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, 2% Pharmalyte, and 1 mM benzamidine)] was extracted for 1 h, and used for two-dimensional gel electrophoresis (2-DE). Isoelectric focusing was performed using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (GE Healthcare, USA). The voltage was increased from 150 to 3500 V over 3 h for sample entry, and the focusing was considered to be complete after 96 kVh. The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel (20×24 cm, 10–16%) for the second dimension was run at 20°C for 1700 Vh. The SDS-PAGE was silver-stained and followed by sensitization with glutaraldehyde (Oakley et al. 1980). To evaluate the change in intensity of each protein spot on the 2-DE gels, quantitative analysis of digitized images was performed using PDQuest software (version 7.0; Bio-Rad, Hercules, CA, USA). The quantity of each spot was normalized by the total intensity of valid spots. Protein spots on each 2-DE gel plate were selected for differences in expression of either over two-fold or less than half of spot intensity compared with the control of distal healthy tissues. When the spot intensity of the control was similar to or the same as that of the background, a minimum intensity value of “1” was assigned and used to calculate a ratio for the expression of increased or decreased proteins. Meanwhile, in this research, we presented the expression levels as the absolute intensity value (after subtracting the background) using PDQuest software in units of parts per million (ppm) to show precise values for the abundance of each protein spot.

Protein digestion and identification

Protein spots were enzymatically digested in gel by the method of Shevchenko et al. (1996) using porcine trypsin ($9 \text{ ng } \mu\text{l}^{-1}$; Promega, Madison, WI, USA) for 9 h at 37°C . For the identification of proteins, samples were analyzed using a 4700 Proteomics Analyzer with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)/TOFTM mass spectrometer (Applied Biosystems, Foster City, CA, USA). Both mass spectrometry (MS) and MS/MS data were acquired with a Nd:YAG laser at 200 Hz repetition rate. At least 4000 laser shots were accumulated for each spectrum. MS/MS mode was operated with 2-keV collision energy. Air was used as the collision gas such that a nominally single-collision condition was achieved. Although the precursor selection has a possible resolution of 200, in these studies of known single component analysis, a resolution of 100 was utilized. Both MS and MS/MS data were acquired using the instrument default calibration, without applying internal or external calibration. Sequence tag was identified via a National Center for Biotechnology Information (NCBI) search using Mascot (Matrix Science Ltd, London, UK) and a European Molecular Biology Laboratory (EMBL) search using MS BLAST (Shevchenko et al. 2001).

Results

The colonial stage of hydrozoans is commonly found on the late-harvested thalli of aquacultured *Saccharina japonica*, often covering much of the kelp surface with a thick sward of horizontal thread-like roots called hydrorhizae. To identify proteins as markers of early colonization or initial cue proteins upon colonization, we separated the proximal, colony-front, and distal healthy tissues to isolate proteins that respond to hydrozoan colonization. Protein isolation from each tissue was replicated and optimized to confirm the differentially displayed protein profiles. For the proximal, colony-front, and healthy tissues, 110, 133, and 78 protein spots, respectively, were detected on 2-DE plates (Figure 2).

Of the 110 spots in the proximal tissues, 109 showed different expression levels compared with distal healthy tissues; 56 spots increased and 53 decreased in protein expression. Of the 56 increased spots, 34 had intensities more than twice those of the distal healthy tissues, and four of these (Nos. 5, 7, 34, and 35 in Figure 2) had intensities more than 1000-fold those in the healthy tissues. Of the 53 decreased spots, the intensities of 36 were less

Figure 2: Two-dimensional gel electrophoresis profiles of late-harvested *Saccharina japonica*. (A) Distal healthy thallus tissue. (B) Thallus tissue proximal to hydrozoan colonies. (C) Thallus tissue at the colony front. The separated proteins were visualized by silver staining. Numbers attached to the arrows refer to the spot numbers listed in Table 1.

than half of those in the healthy tissues, and the intensities of three of these (Nos. 22, 36, and 37) were less than one thousandth of those in the healthy tissues. These seven spots (four increased and three decreased) that differed markedly in intensity between tissues appeared constantly in replicated experiments for protein analysis. We identified the seven proteins in a database search of proteins from algae, land plants, and bacteria. Table 1 lists the identities of these seven proteins with their molecular weight (MW), isoelectric point (pI) values, and functions.

Table 1: Proteins identified in the thallus tissue proximal to hydrozoan colonies, in tissue at the colony front, and in distal healthy tissue of late-harvested *Saccharina japonica*.

Spot no.	Protein name	Taxonomy	pI	Mass (kDa)	Accession ID (NCBI)	Mascot score	Accession ID (EMBL)	MS BLAST score	Spot intensity (ppm)	Function
Proteins highly increased in the thallus tissue proximal to hydrozoan colonies, but not detected in healthy tissue										
5	Actin	<i>Saccharina japonica</i> / <i>Crassostrea gigas</i>	4.9	56	gi 77745053	161	EKC38741	1846	1344	Cytoskeleton
7	CIPK20 (CBL-interacting serine/threonine-protein kinase 20)	<i>Arabidopsis thaliana</i>	4.9	86	gi 218186108	85	ABA94760	1567	3170	Signal transduction
34	SIPA11.1 (signal-induced proliferation-associated 1-like protein 1)	<i>Heterocephalus glaber</i>	4.6	86	gi 512949794	79	–	–	2580	Proliferation
35	SIPA11.2 (signal-induced proliferation-associated 1-like protein 2)	<i>Camelus ferus</i>	5.2	85	gi 560915741	86	–	–	2350	Proliferation
Proteins found mostly in the distal healthy tissue, but not detected in thallus tissue proximal to hydrozoan colonies										
22	NEK2 kinase (serine/threonine-protein kinase Nek2 isoform X1)	<i>Pelodiscus sinensis</i>	4.7	28	gi 558165793	79	XP_006123907	2762	1592	Signal transduction
36	CaMK2N2 (calcium/calmodulin-dependent protein kinase II inhibitor 2)	<i>Pantholops hodgsonii</i>	4.4	17	gi 556772980	48	XP_005981651	698	1842	Signal transduction
37	GK25369 (DNA replication factor Dna2)	<i>Drosophila willistoni</i>	4.5	32	gi 195446960	123	XP_002071002	8105	1771	DNA replication
Proteins highly increased in the tissue at the colony front, but not detected in distal healthy tissue										
7	CIPK20 (CBL-interacting serine/threonine-protein kinase 20)	<i>Arabidopsis thaliana</i>	4.9	86	gi 218186108	85	ABA94760	1567	1969	Signal transduction
35	SIPA11.2 (signal-induced proliferation-associated 1-like protein 2)	<i>Camelus ferus</i>	5.2	85	gi 560915741	86	–	–	2128	Proliferation
41	ATPase β (chloroplast ATP synthase CF1 beta chain)	<i>Undaria pinnatifida</i> / <i>Saccharina japonica</i>	5.0	65	gi 560915741	168	YP_006639069	3328	5964	ATP synthesis
42	HECT domain protein (ubiquitin-transferase containing protein)	<i>Chlamydomonas reinhardtii</i> / <i>Eimeria brunetti</i>	5.5	80	gi 159465371	83	CDJ46073	961	2979	Defense system
43	ADA (adenosine deaminase)	<i>Dendroctonus ponderosae</i> / <i>Aedes aegypti</i>	5.6	80	gi 332374166	76	XP_001651061	714	3988	Nucleic acid metabolism
44	Kinesin	<i>Eimeria mitis</i> / <i>Eimeria maxima</i>	6.5	68	gi 557177808	84	CDJ56258	6747	3549	Mechanical work
Proteins found mostly in the distal healthy tissue, but not detected in tissue at the colony front										
23	Transmembrane protein	<i>Burkholderia multivorans</i>	4.7	32	–	–	Q845W1	201	324	Signaling response
36	CaMK2N2 (calcium/calmodulin-dependent protein kinase II inhibitor 2)	<i>Pantholops hodgsonii</i>	4.4	17	gi 556772980	48	XP_005981651	698	1842	Signal transduction
37	GK25369 (DNA replication factor Dna2)	<i>Drosophila willistoni</i>	4.5	32	gi 195446960	123	XP002071002	8105	1772	DNA replication
45	MSP (photosystem II manganese-stabilizing protein)	<i>Ectocarpus siliculosus</i>	5.3	35	gi 299471042	90	CBN78902	2346	4417	Photosynthesis

The spot intensities of proteins in the proximal, front and healthy tissues are presented after subtracting the background.

Upon searching the NCBI and EMBL databases, seven and five proteins, respectively, were confidently identified. Five proteins were present in both databases. The seven identified proteins were actin, calcineurin B-like protein (CBL)-interacting serine/threonine-protein kinase 20 (CIPK20), signal-induced proliferation-associated 1-like protein 1 (SIPA1L1), signal-induced proliferation-associated 1-like protein 2 (SIPA1L2), serine/threonine-protein kinase Nek2 isoform X1 (NEK2 kinase), calcium/calmodulin-dependent protein kinase II inhibitor 2 (CaMK2N2), and DNA replication factor Dna2 (GK25369). Among the identified proteins, four increased proteins (actin, CIPK20, SIPA1L1, and SIPA1L2) were primarily expressed in the proximal tissues (spot intensities were 1344, 3170, 2580, and 2350 ppm, respectively) and not detected in the distal healthy tissues (Figure 3). Three decreased proteins (NEK2 kinase, CaMK2N2, and GK25369), which were present in the healthy tissues (spot intensities of 1592–1842 ppm) but were not present in the proximal tissues, were significantly down-expressed in the proximal tissues (Figure 4). Of the seven proteins identified from a homology-based cross-species database, we found three proteins related to signal transduction, two proteins related to proliferation, one protein related to the cytoskeleton, and one protein related to DNA replication.

Of the 133 spots in the colony-front tissues, 67 spots increased, 60 decreased, and 6 were unchanged (Figure 2).

Of the 67 increased spots, the intensities of 53 were more than twice those in the distal healthy tissues, and six of these spots (Nos. 7, 35, and 41–44) had intensities more than 1000-fold those in the healthy tissues. Of the 60 decreased spots, the intensities of 45 were less than half those in the healthy tissues, and four of these spots (Nos. 23, 36, 37, and 45) were less than one thousandth of the intensity of those in the healthy tissues. The 10 spots (six increased and four decreased) that differed most markedly in intensity between tissues appeared constantly in replicated experiments for protein analysis. A database search of proteins from algae, land plants, and bacteria identified the 10 proteins. Table 1 lists the identities of these 10 proteins with their MW, pI, and functions. Searches of the NCBI and EMBL databases identified nine proteins in each database, of which eight proteins were present in both databases. The 10 identified proteins were CIPK20, SIPA1L2, chloroplast ATP synthase CF1 beta chain (ATPase β), ubiquitin-transferase containing protein (HECT domain protein), adenosine deaminase (ADA), kinesin, transmembrane protein, CaMK2N2, GK25369, and photosystem II manganese-stabilizing protein (MSP). Of these identified proteins, the six with increased expression (CIPK20, SIPA1L2, ATPase β , HECT domain protein, ADA, and kinesin) were mostly expressed in the colony-front tissues (spot intensities were 1969, 2128, 5964, 2979, 3988, and 3549 ppm, respectively) and not detected in the

Figure 3: Sections of two-dimensional gels showing the proteins (arrows) that increased most in the thallus tissue proximal to hydrozoan colonies or in the thallus at the colony front, but were not detected in the distal healthy thallus tissue. The separated proteins were visualized by silver staining. (A) Distal healthy thallus tissue. (B) Thallus tissue proximal to hydrozoan colonies. (C) Thallus tissue at the colony front.

healthy tissues (Figure 3). The remaining four proteins (transmembrane protein, CaMK2N2, GK25369, and MSP) were present (324–4417 ppm) in the healthy tissues but not present in the colony-front tissues (Figure 4). Of the 10 proteins identified from a homology-based cross-species database, we identified two proteins related to signal transduction, and one protein each related to proliferation, ATP synthesis, defense system, nucleic acid metabolism, mechanical work, signaling response, DNA replication, and photosynthesis.

Actin and SIPA1L1 increased only in the proximal tissues with spot intensities of 1344 and 2580 ppm, respectively, but were not detected in the healthy and colony-front tissues. Therefore, actin and SIPA1L1 can be used as specific markers for the early warning response of *Saccharina japonica* to hydrozoan colonization. The proteins ATPase β , HECT domain protein, ADA, and kinesin increased only in the colony-front tissues with spot intensities of 5964, 2979, 3988, and 3549 ppm, respectively, and were not detected in the healthy and proximal tissues. In particular, proteins CIPK20 and SIPA1L2 increased strongly in both the proximal tissues (3170 and 2350 ppm, respectively) and colony-front tissues (1969 and 2128 ppm, respectively), but were rare in the distal healthy tissues. CIPK20 and SIPA1L2 may also serve as hydrozoan colonization cue proteins induced in the colony-front and proximal sections of the colonized *S. japonica* thalli. NEK2 kinase decreased only in the proximal tissues, and was found mostly in the colony-front or healthy tissues. Therefore, NEK2 kinase can be used as a specific repression marker for the early warning response of *S. japonica* to hydrozoan colonization. The proteins transmembrane protein and MSP were decreased in the colony-front tissues, and

found mostly in the healthy tissues. In particular, proteins CaMK2N2 and GK25369 were decreased in both the colony-front and proximal tissues, but found mostly in the healthy tissues (1842 and 1772 ppm, respectively). The proteins CaMK2N2 and GK25369 may also serve as hydrozoan colonization cue proteins reduced in the colony-front and proximal sections of the colonized *S. japonica* thalli.

Discussion

The goal of proteomics is to analyze the various sets of proteins (proteomes) at different times under defined environmental conditions to highlight differences between them. One of the most practical applications of proteomics is the identification and characterization of target proteins as opposed to entire proteomes. This type of proteomics is referred to as functional proteomics (Graves and Haystead 2002). Proteomic analysis has been used to reveal complex plant-insect interactions that help identify candidate genes involved in the defense response of plants to herbivory (Wu and Baldwin 2010). Most research on plant-insect interactions thus far has mainly focused on the proteomics and genomics of the late events of plant defense. The early events (i.e. recognition and triggering of signal transduction) are, on the other hand, poorly understood (Fürstenberg-Hägg et al. 2013). Protein profiling using proteomics is also used to identify biomarkers that are up- or down-regulated in response to parasite colonization. We examined the proteomic profiles of late events of *Saccharina japonica* in response to bryozoan (Getachew et al. 2014) and hydrozoan (Getachew et al.

Figure 4: Sections of two-dimensional gels showing the proteins (arrows) that decreased during hydrozoan colonization. The separated proteins were visualized by silver staining. (A) Distal healthy thallus tissue. (B) Thallus tissue proximal to hydrozoan colonies. (C) Thallus tissue at the colony front.

2016) infestation. We identified early events in *S. japonica* tissues proximal to and beneath the front of bryozoan colonies (Getachew et al. 2018). Early events in response to bryozoans produced higher numbers of increased protein spots (99) in tissues proximal to the colony, than occurred in response to hydrozoan infection (56 protein spots increased). The thallus tissues beneath bryozoan colonies had elevated levels of potassium, iodine, and docosahexaenoic acid compared with healthy tissues (Getachew et al. 2015b), whereas the tissues beneath hydrozoan colonies had higher levels of selenium, iodine, and docosahexaenoic acid than healthy tissues (Getachew et al. 2015a). As of yet, however, no study has evaluated the early events of epiphytic hydrozoan infection on the proteomic profiles of the *S. japonica* thallus proximal to and beneath the front of hydrozoan colonies.

In higher plants, the sagebrush *Artemisia tridentata* becomes more resistant to herbivores following exposure to volatile cues from neighbors damaged by clipping or natural herbivores (Karban and Shiojiri 2009). Cue compounds are required for systemic-induced resistance among branches on individual sagebrush plants, while vascular connections are insufficient. Hydrozoans represent an extremely diverse group of mostly colonial forms. Planktotrophic larvae of the stoloniferous hydrozoans settle on kelp thalli, resulting in widespread colonies covering the surface of the seaweed. Epiphytic colonization by hydrozoans has a ripple effect (i.e. small colonies on the thalli expand outwards incrementally). Therefore, we postulated that the colony-front and proximal tissues might reflect the early stage of the infection. Planula larvae are too small to detect on dark brown *Saccharina japonica* thalli at the time of settlement before or after their metamorphosis. Although these colony-front and proximal tissues do not represent direct materials for detecting early planula infection, they (as colony-neighboring tissues) may respond to and produce early event signals in response to epiphytic infection. Although no cues from colonized or damaged seaweed tissues are known, we postulated that early signals may be provided by the hydrozoans or colonized tissues. To examine the early signals of colonization, we prepared tissue sections from the colony-front and proximal tissues and compared them with the distal healthy tissues (control).

The cytoskeleton protein actin increased strongly in the tissue proximal to hydrozoan colonies but it was nearly absent in the colony-front and healthy tissues. Plants resist pathogens by preventing their penetration of the epidermis, a key component of basal defense. This is achieved by rapid re-organization of actin microfilaments

(Hardham et al. 2007). In plants, the actin component of the cytoskeleton coordinates processes such as development and responds to abiotic and biotic stimuli (McCurdy et al. 2001). Actin plays a role in the formation of a physiological barrier at the site of infection (Janda et al. 2014). Actin is also highly expressed in tissues proximal to bryozoan colonies (Getachew et al. 2018).

The signal-induced protein SIPA1L1 increased in large quantities only in the tissue proximal to hydrozoan colonies and was not detected in the colony-front and healthy tissues. SIPA1L1, or signal-induced proliferation-associated protein, is a GTPase-activating protein (GAP) involved in mitogen-induced cell cycle regulation (Hunter and Crawford 2006). GAPs are a family of regulatory proteins whose members bind to activated G proteins and stimulate their GTPase activity, terminating the signaling event. Overexpression of GTPase-activating protein 1 (OsGAP1) in transgenic rice (*Oryza sativa*) raised the basal transcriptional levels of all defense marker genes tested (Chern et al. 2005). High expression of SIPA1L1 in the early phase of colonization (i.e. in the proximal tissues) may indicate that the defense strength of the seaweed against colony attachment is enhanced during the early stages. Therefore, this signal-induced protein could represent a marker specific for the proximal tissues. SIPA1L1 was also highly expressed in the early phase of bryozoan colonization (Getachew et al. 2018). SIPA1L2, another member of the signal-induced proliferation-associated protein family, increased strongly in the colony-front and proximal tissues. SIPA1L2 represents a potential marker specific for these tissues. High expression of SIPA1L1 and SIPA1L2 in the early stage of hydrozoan colonization may represent a response to, and defense against, colonization by the seaweed. Further studies should characterize these proteins of *Saccharina japonica* in detail and determine the underlying molecular mechanisms of how these two similar proteins respond to epiphytes differently.

CIPK20 (CBL-interacting serine/threonine-protein kinase 20, also known as SOS2-like protein kinase PKS18; EC:2.7.11.1) increased strongly in the proximal tissues and also increased in large quantities in the colony-front tissues, but was not detected in the healthy tissues. The plant family of serine/threonine protein kinases (e.g. CIPKs or PKS) and their activators function together in decoding calcium signals caused by different environmental stimuli (Chaves-Sanjuan et al. 2014). The CBL-CIPK signaling system provides a fast and efficient method of molecular design breeding of crop plants to enhance tolerance to abiotic stresses (Li et al. 2009). CIPK20 was also highly expressed in the thallus tissue

proximal to bryozoan colonies (Getachew et al. 2018). Thus, CIPK20 may increase kelp tolerance to abiotic and biotic stresses.

The protein ATPase β increased strongly only in the colony-front tissues and was not detected in the proximal and healthy tissues. This chloroplast ATP synthase is integrated into the thylakoid membrane, where the dark reactions of photosynthesis and ATP synthesis take place by the proton motive force. Each β subunit is anchored to the membrane through a single transmembrane helix and interacts with the α subunit, another stator subunit (DeLeon-Rangel et al. 2013). The overall structure and catalytic mechanism of the chloroplast ATP synthase are almost the same as those of the mitochondrial enzyme. The ATPase was also highly expressed in tissues proximal to and beneath the front of bryozoan colonies (Getachew et al. 2018). In animals, ATPase activity is involved in lipid transfer and/or in the lipophorin shuttle mechanism. Lipophorin, the main lipoprotein in insect circulation, cycles among peripheral tissues to exchange its lipid cargo at the plasma membrane of target cells (Fruttero et al. 2014). Additional research is necessary to address whether seaweed ATPase activity is involved directly or indirectly in lipid transfer and/or the lipophorin shuttle mechanism of epiphytes.

NEK2 kinase decreased only in the proximal tissues. The Nek family of serine/threonine kinases functions in cell cycle regulation and initiates the separation of centrosomes at G2/M (Westwood et al. 2009). In animal tumor cells, the depletion of NEK2 leads to an apparent arrest in cell proliferation and an increase in apoptosis, possibly as a result of mitotic errors (Hayward and Fry 2006). Thus, early in infection, the seaweed may respond to cease cell proliferation. NEK2 kinase was also significantly decreased by bryozoan epiphytes (Getachew et al. 2018). Therefore, the NEK2 kinase may be used as a specific repression marker for the early warning response of *Saccharina japonica* to epiphyte colonization. MSP decreased only in the colony-front tissues. MSP is required for photosystem II assembly and photoautotrophy in higher plants (Yi et al. 2005). Additionally, hydroid colonies reduced *S. japonica* tissue viability by 77% compared with healthy tissues (Getachew et al. 2015a). CaMK2N2 was nearly absent in both the colony-front and proximal tissues. Calcium, a universal secondary messenger in plants, plays vital roles in response to various signals, including light, mechanical disturbance, abiotic stress, and pathogen elicitors (Zhang and Lu 2003). Cytosolic calcium is regulated by Ca^{2+} -binding proteins, including CaMK in signal transduction and stress resistance. Plants overexpressing these proteins can increase their stress tolerance. Thus, down-regulation of the CaMK inhibitor CaMK2N2 in the

colony-front and proximal tissues may help the seaweed increase stress resistance. This protein was also weakly expressed in tissues under hydrozoan colonies (Getachew et al. 2016).

In conclusion, the protein SIPA1L2 increased strongly in both the thallus tissues proximal to the hydrozoans and beneath the colony front. Proteins HECT domain protein, ADA and kinesin increased strongly in tissues beneath the colony front. These 4 proteins were not increased in response to bryozoan infection. Meanwhile, the proteins SSP15, Rpl1P, and SRSF increased strongly in the tissues near bryozoan colonies, but were not expressed in response to hydrozoan infection. Actin, CIPK20, SIPA1L1 and ATPase β increased strongly near both hydrozoan and bryozoan colonies. As hydrozoan colonies grow, hydromedusae from the colony front spread out and cover the whole of the *Saccharina japonica* thallus, and identifying these initial colonization-induced signals would provide markers for the early events of epiphytic infestation or for induced defense responses from thallus tissue proximal to the epiphyte and under the colony front. By increasing their expression, the early cue proteins related to defense mechanisms could help to improve the resistance of *S. japonica* to epiphyte infestation.

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