



Article Identification, Characterization, and Expression Analysis of Spondin-Like and Fasciclin-Like Genes in *Neopyropia yezoensis*, A Marine Red Alga

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Abstract: Extracellular matrix (ECM) proteins play crucial roles in the regulation of cell proliferation and differentiation. We identified homologous genes encoding ECM proteins that are known to associate with integrins in animal cells in red macroalga *Neopyropia yezoensis*. Four genes encoding spondin domain-containing proteins (NySPLs) and eight genes encoding fasciclin domain-containing proteins (NyFALs) from *N. yezoensis* were selected for bioinformatics and expression analysis in order to obtain insights into the roles of ECM proteins for the life cycle. NySPLs had eight β -strands with two contiguous α -helices, which were similar to those of the F-spondin domain of animals. NyFALs had conserved H1 and H2 motifs and a YH motif between the H1 and H2 regions. Quantitative reverse transcription polymerase chain reaction showed that *NySPL1–3* and *NyFAL8* transcripts were highly accumulated in mature gametophytes that formed the spermatia. Furthermore, expressions of all *NySPLs* were upregulated in response to the ethylene precursor 1-aminocylopropane-1-carboxylic acid that induces gametogenesis. *NyFAL1*, *4* were highly expressed in sporophytes, whereas *NyFAL2*, *3*, *5*, *6*, and *7* were overexpressed in gametophytes, especially at the vegetative stage. These findings facilitate future research on ECM architecture in the unique life cycles of red macroalgae.

Keywords: extracellular matrix proteins; fasciclin; Neopyropia; red algae; spondin

1. Introduction

The extracellular matrix (ECM) not only provides structural support for organs and tissues in the form of basement membranes but also regulates cell–cell communication and signaling [1,2]. ECMs from macroalgae, which are commonly referred to as the cell wall, are complex assemblages of cellulose, various hemicelluloses, and unique sulfated polysaccharides (ex. agars and carrageenans) [3]. With regard to the research on ECMs, many reports focus on the ECM polysaccharides, because they are main components of cell wall structures in addition to useful materials for gel-forming agents, cosmetics, biofuels, nutraceuticals, and pharmaceuticals [4].

ECM proteins are among the most important ECM components that regulate cell proliferation and differentiation by binding to multiple interacting partners, such as other ECM proteins and signal receptors [2,5]. In animal cells, integrins, which are a superfamily of cell adhesion receptors, trigger various signal transduction events that modulate cell behavior, such as adhesion, proliferation, polarity, differentiation, and gene expression, by binding to ECM ligands [6,7].

The marine red algae Bangiophyceae, such as *Neopyropia*, *Porphyra*, and *Pyropia*, is one of the most important marine aquaculture crops harvested for human food. Bangiophyceae has a heteromorphic life cycle, with alterations between its blade gametophyte and filamentous sporophyte [8,9] (Figure 1). A previous study showed the gametophyte and sporophyte of Bangiophyceae differ markedly in the composition of the ECM polysaccharides [10]. In addition, the reproductive regions of Bangiophyceae gametophyte differ



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). from vegetative regions in the sugar composition of ECMs [11–13]. Thus, ECM remodeling appears to occur during the life cycle of Bangiophyceae species. However, in contrast to ECM polysaccharides, our knowledge of the role of ECM proteins during the unique life cycle remains limited in spite of the availability of Bangiophyceae genome data sets [14–16].



Figure 1. Life cycle of the marine red alga *Neopyropia yezoensis*. The mature gametophytes of Bangiophyceae form male (spermatia) and female gametes (carpogonia) during sexual reproduction. After fertilization occurs, released zygotes (called carpospores) grow into filamentous sporophytes (called *Conchocelis*). Mature sporophytes subsequently produce conchospores that are released from the conchosporangia and settle on the substratum, where they germinate to form new gametophytes. In addition to the sexual life cycle, some species, such as *Neopyropia yezoensis*, have an asexual life cycle in which asexual spores termed as archeospores (or monospores) are produced, which subsequently reproduce gametophytic thallus. VGA: vegetative gametophytes formed vegetative cells only; MGA: mature gametophytes formed sexual cells; VSP: vegetative sporophytes formed vegetative cells only; MSP: mature sporophytes formed conchosporangia.

Bangiophyceae does not appear to possess integrin adhesion and signaling systems similar to animals. However, upon exploring the genomic data of *N. yezoensis*, we found homologs of ECM proteins that interact with integrins in animal cells, such as F-spondin and fasciclin. The spondin family, including F-spondin and Mindin, are the molecules that are attached to the ECM [17]. The F-spondin molecule consists of approximately 800 amino acids that contains domains of homologous to reelin, FS domain, and multiple TSR repeats (five in invertebrate and six in vertebrate) [18], while Mindin contains an FS domain and one TSR domain [19]. Structural studies suggested that the FS domain, which exhibits a homology structure similar to that of the C2 domain functions as membrane-targeting modules through Ca²⁺-dependent [20]. The F-spondin family, which was identified in animal species, including rats [21], Xenopus [22], Drosophila [19], and zebrafish [18], influences the patterning of the nervous system by promoting adhesion and outgrowth of commissural axon [23]. In contrast, members of the fasciclin I family of proteins (FAS1), which are ancient extracellular proteins, are found in many vertebrates, invertebrates, plants, and microorganisms [24]. FAS1 also participates in cell adhesion and communication for diverse biological processes, such as axon guidance, morphogenesis, and cell proliferation [25,26]. Mammalian FAS1 are thought to bind to different types of integrins that mediate mechanical contact between the cytoskeleton and the ECM as well as transduce intracellular signals using numerous associated proteins [24,27]. In higher plants, FAS1 domain proteins, named FLAs, were identified as a group of hydroxyproline-rich glycoproteins (HRGPs) called AGPs [28]. FLAs have a plethora of biological roles, such as the maintenance of cell wall architecture, regulation of male gametophyte and seed development, and enhancement of root growth and shoot regeneration [29–34].

Fossil records (ca.1000 Mya) of Bangiomorpha, which closely resemble the extant Bangiophyceae, provide the oldest evidence of sexually reproducing eukaryotes, since it is possible to observe both spore and gamete formation [35,36]. The findings imply that Bangiophyceae species possess a primitive ECM remodeling mechanism for progression of the life cycle. In the present study, we identified and characterized candidate ECM genes including four spondin-like (*NySPLs*) and eight fasciclin-like (*NyFALs*) genes in *N. yezoensis* that is harvested to produce nori in Japan toward our understanding of the role of ECM for ancestral developmental and reproductive processes.

2. Materials and Methods

2.1. Algal Materials and Treatments

Leafy gametophytes and filamentous sporophytes of the *N. yezoensis* strain TU-1 [37] were cultured at 15 °C in sterile vitamin-free Provasoli's enriched seawater (PES; [38]) under a 10 h light/14 h dark photoperiod provided using cool-white, fluorescent lamps at a light intensity of 40 μ mol photons m⁻² s⁻¹, as described previously [39].

Vegetative gametophytes that microscopically formed only vegetative cells and mature gametophytes that produced clusters of spermatangia were frozen with liquid nitrogen and stored at -80 °C until the RNA was extracted for quantitative reverse transcription polymerase chain reaction (qRT-PCR). Similarly, vegetative sporophytes that formed only vegetative cells and mature sporophytes that produced conchosporangia were also frozen and stored at -80 °C. Observation of vegetative and reproductive cells was performed by a Leica DM 5000 B microscope.

In our previous study, gametophytes treated with Hsp90-inhibiting drugs, such as geldanamycin (GA) and radicicol (RAD), induced the formation of archeosporangia [40]. Thus, to induce an asexual life cycle, vegetative gametophytes were cultured at 15 °C under a 10 h light/14 h dark photoperiod in a PES medium containing 5 μ M RAD (Cayman Chemical Company) that was prepared by dissolving RAD in dimethyl sulfoxide (DMSO) for 7 days. Simultaneously, the control experiment was conducted using a DMSO-containing culture at concentrations corresponding to the same volume of the reagent. Alternatively, to induce sexual life cycle, vegetative gametophytes grown to a blade length of 20–30 mm were used for transcriptional analyses under 1-aminocylopropane-1-carboxylic acid (ACC) treatments. Then, for ACC treatments, cultured algae were treated with PES medium containing 50 μ M ACC (Tokyo Chemical Industry, Tokyo, Japan). The gametophytes that induced asexual and sexual reproduction by the reagents were also frozen and stored at -80 °C.

2.2. Transcriptional Analysis of NySPL and NyFAL Genes

RNA extraction and qRT-PCR were performed as described previously [41]. Total RNA from gametophytes and sporophytes (Fresh weight: 0.05–0.1 g) was separately extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in liquid nitrogen with a mortar and pestle, following the manufacturer's instructions. Then, extracted RNA was purified using the TURBO DNA-free kit (Invitrogen/Life Technologies, Carlsbad, CA, USA) to obtain DNA-free RNA samples. Afterward, first-strand cDNA was synthesized from 0.5 μ g total RNA using PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan). For subsequent qRT-PCR, obtained cDNA was diluted 10-fold and 1.0 μ L of the diluted cDNA was used as a template in a 20-microlitre reaction volume. qRT-PCR was performed using the KOD SYBR[®] qPCR Mix kit (TOYOBO, Osaka, Japan), following the manufacturer's instructions. Real-time PCR was then conducted using a LightCycler[®] 480

System (Roche Diagnostics, Basel, Switzerland) under the following conditions: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 31 s at 55 °C. Expression levels for each *NySPL* and *NyFAL* gene were calculated using the $2^{-\triangle \triangle Ct}$ method [42], and the levels were normalized to the level of the transcription elongation factor 1 (*PyElf1*) gene [43]. Finally, relative expression levels of the examined genes were calculated as a ratio of the mRNA level to the transcription level at the vegetative gametophyte stage at 0 day after ACC treatment. qRT-PCR was conducted in triplicate. Table S1 lists the primers used in this study.

2.3. Bioinformatic Analysis

After the functional annotation of draft N. yezoensis genome sequence [14] was analyzed using Blast2GO software (https://www.blast2go.com/) (accessed on 8 June 2021) with an E value cutoff of 1×10^{-4} [44], four genes encoding spondin domain-containing proteins (NySPLs) and eight genes encoding fasciclin domain-containing proteins (Ny-FALs) were identified by key word search on the annotated genome. Based on these sequences, complete open reading frames were retrieved from N. yezoensis genome sequence data (ASM982973v1). The protein sequences from NySPLs and NyFALs were blasted against NCBI database using BLASTp with the default parameter. Then, their isoelectric points (pIs) and molecular weights (Mws) were calculated using ExPASy (http: //web.expasy.org/cgi-bin/compute_pi/) (accessed on 5 August 2021) [45]. Conserved domains were confirmed using the "ScanProsite" feature under ExPASy (http://tw.expasy. org/tools/scanprosite/) (accessed on 5 August 2021), and the PROMALS3D multiple protein sequences alignment program (http://prodata.swmed.edu/promals3d/promals3 d.php) (accessed on 21 August 2021) [46] was used to align the deduced amino acid sequences. PSORT II (https://psort.hgc.jp/form2.html) (accessed on 25 August 2021) [47] was used to predict the algae's subcellular localization, and the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) (accessed on 25 August 2021) [48] was used to predict N-glycosylation sites using artificial neural networks that can examine Asn-Xaa-Ser/Thr sequences.

3. Results

3.1. Characterization of Spondin Domain-Containing Proteins

The F-spondin family of proteins includes both F-spondin and M-spondin/Mindin that share similarities in their F-spondin (FS) domains and thrombospondin repeat (TSR) domains [18,19]. In this study, four genes encoding FS domain-containing proteins were identified from the *N. yezoensis* genome sequence and were named NySPL1–4 (spondin-like proteins) (Figure S1). Details of NySPL proteins, including protein length, molecular weight, pI, and predicted subcellular localizations are listed in Table 1. The lengths of the NySPL proteins ranged from 223 to 232 amino acid residues, whereas predicted pI values ranged from 5.13 to 10.37. NySPLs had 45–49% similarity of the FS domain of human F-spondin. Furthermore, BLAST analysis showed that the protein sequences of NySPLs exhibited a 45–72% identity with FS domain-containing proteins from *Porphyra umbilicalis*. All four NySPLs lacked a TSR domain.

Table 1. Characteristics of NySPL proteins identified in Neopyropia yezoensis genome.

Name	Contig ID	Size (aa)	pI	Mw (KDa)	Predicted Location
NySPL1	contig_17719_g4350	228	5.78	24.64	extracellular
NySPL2	contig_23899_g5890	223	10.37	24.18	extracellular
NySPL3	contig_29619_g7276	232	9.69	24.46	extracellular
NySPL4	contig_33564_g8106	224	5.13	22.55	extracellular

aa-amino acid; pI-isoelectric point; Mw-Molecular weight.

Secondary structure analysis revealed that all four NySPLs had eight β -strands with two contiguous α -helices, which were similar to those of the FS domain from animals (Figure 2A). Multiple sequence alignment showed that all NySPLs had two highly conserved sequences, i.e., "PSPDW" and "DAGTD", in the FS domain. These conserved sequences comprised three aspartic acid residues, which functioned as a solvent-accessible Ca²⁺-binding site [20]. The results also showed that the N-linked glycosylation site in the FS domain from humans was conserved in NySPL1, 3. Likewise, a putative N-glycosylation site was detected within other NySPL proteins. However, the integrin-binding LEV triplet found in the FS domain of human, mouse, and rat mindins [49] was not conserved in NySPLs.

3.2. Characterization of Fasciclin Domain-Containing Proteins

Although the overall sequence conservation among FAS1 domains is low, two conserved sequence motifs called H1 and H2 exist [50]. Another conserved central [Phe/Tyr]-His (YH) motif, with approximately 10 amino acids in each conserved region has also been identified [50]. Prokaryotic representatives typically contain a single FAS1 domain, whereas many eukaryotic FAS1 family members contain two or more FAS1 domains in tandem [24]. In this study, eight genes encoding FAS1 domain-containing proteins were identified from the *N. yezoensis* genome sequence and were named NyFAL1–8 (fasciclin-like protein) (Figure S1). Details of the identified NyFAL proteins are listed in Table 2.



(A)

Figure 2. Cont.

			H1							
NyFAL1	223	-RK <mark>I</mark> IAEVVASTDDFSL <mark>I</mark>	LAALEAADLVDAVADRDASLTV <mark>FAP</mark> TND <mark>AF</mark> IR <mark>L</mark> SRTLGLYEDNVKPDEALAY	291						
NyFAL2	84	-PQ <mark>T</mark> IADIVAGNDDFSV <mark>I</mark>	LTALQAASLVETLDDESASL <mark>TV</mark> FAP <mark>TNG</mark> AFLS <mark>L</mark> AQTLGYTGDNVDGVFAF	150						
NyFAL3	39	-PP <mark>T</mark> LAALVAKRDDLSI	EEALKLTGLWDTLAKANTSSTL <mark>FAP</mark> NDA <mark>AF</mark> VD <mark>L</mark> SFWLKEGGSDKAGALKT	105						
NyFAL4	423	-PA <mark>T</mark> VAAIISAADGFAV <mark>I</mark>	GEALEASGLLAALGHPSAVL <mark>TV</mark> FAP <mark>TDA</mark> AFVR <mark>L</mark> ARVLGYAAASDHPTPIFNF	491						
NyFAL5	54	-GN <mark>T</mark> IHENLKAAGAFKT <mark>I</mark>	IAAAAASDAYGAGTKGEFMV <mark>FAP</mark> SDA <mark>AF</mark> AR <mark>L</mark> RPGTLEALLA	111						
NyFAL6	73	-TS <mark>T</mark> IVETLADVPVFST <mark>I</mark>	RSLLAETGLDYELSKA-GPWTL <mark>FAP</mark> TDE <mark>AF</mark> AG <mark>L</mark> LEPHSFSTLA	131						
NyFAL7	49	GPP <mark>T</mark> ILDVLQADDDFSI <mark>I</mark>	AYTVDAAGVGPLLGDPAATL <mark>TV</mark> FAPTDC <mark>AF</mark> MA <mark>L</mark> ARALGFNVGTKGAARRF	116						
NyFAL8	29	-AQ <mark>T</mark> IAEIATGNANLTL <mark>I</mark>	VQALNDTGLVPAVANASASLTV <mark>FAP</mark> TND <mark>AE</mark> LS <mark>L</mark> AQVLGYSGNDADEAYGA	95						
YH										
NyFAL1	292	IL-KKLTTLGDGDPLP	PL <mark>E</mark> KRILTY <mark>H</mark> VLPREVSTRQLAGKTVQTLE <mark>C</mark> GRLHIRPWLRIVDAAPA	353						
NyFAL2	151	LVEALTGLSNGGNPVF	PL <mark>L</mark> TSVLRF <mark>H</mark> VVGSTVSSTELVSTGFFEPLE <mark>G</mark> PAVVLADDGRTLYDFAPGV	216						
NyFAL3	106	VLGELGLIAGFPKVDPVS	S <mark>L</mark> AAFLEF <mark>H</mark> MTDGAVSLKDLVAARGYTSVN <mark>G</mark> ERVKLGADNKTLVDTAT	171						
NyFAL4	492	LI-TTFTRMGGGNPLF	PI <mark>L</mark> QRILSY <mark>H</mark> IVPGRLGTRDLAGNTVRTVE <mark>G</mark> GLIAVRQWLRVIDLAPA	553						
NyFAL5	112	PGKEA	AT <mark>L</mark> RAIVKR <mark>H</mark> VVPGNVLTLQKMKGV-GYLEGVE <mark>G</mark> GPLSYEALGGVIRVGN	164						
NyFAL6	132	GLLRPENRR1	'E <mark>L</mark> RRVLAY <mark>H</mark> ILQGSHPAGSVCAAGTVTGPTLA <mark>G</mark> VDLTVMGYNKKVTAG	189						
NyFAL7	117	IFDALAGLDGGGDPAF	PL <mark>L</mark> ASILKY <mark>H</mark> VTAGAVAAEALVAAGGFQPLQ <mark>G</mark> PRVRLGADNKTLVDAAP	180						
NyFAL8	96	IGAALANLSPDGDAVE	PL <mark>E</mark> RDVLLY <mark>H</mark> VAAEEVMSGALLAAG-GYESLLN <mark>G</mark> TRVELADDNLTLIDAAP	161						
			H2 ^{↑↑}							
NyFAL1	354	VRMNALIHPG <mark>D</mark> VAAA <mark>NG</mark> V	/I <mark>⊞</mark> VID <mark>€V</mark> L 380							
NyFAL2	217	ADPMLVVDRLDIQAS <mark>NG</mark> V	/I <mark>H</mark> VITG <mark>V</mark> LL- 243							
NyFAL3	172	SIDDVKILETDIAAS <mark>NG</mark> V	/M <mark>H</mark> IVGG <mark>V</mark> M 197							
NyFAL4	554	VRMNAQIAPGDLGAS <mark>NG</mark> V	/V <mark>H</mark> VIDA <mark>V</mark> LL- 580							
NyFAL5	165	ALVEQNRSNIECS <mark>NG</mark> I	I <mark>H</mark> VIDS <mark>VL</mark> VP 190							
NyFAL6	190	SARVVKADVAAS <mark>NG</mark> V	/I <mark>H</mark> VVNS <mark>VL 212</mark>							
NyFAL7	181	SVEDPVLVMT <mark>D</mark> KRVS <mark>NG</mark> V	/I <mark>H</mark> TLSG <mark>V</mark> LL- 207							
NyFAL8	161	LVDDPMITQPDIDAS <mark>NG</mark> V *	/I <mark>H</mark> LISG <mark>V</mark> L 186							

(B)

Figure 2. Sequence characteristics of NySPLs and NyFALs. (**A**) Sequence alignment of spondin-like proteins (NySPLs) identified in *Neopyropia yezoensis*. The PROMALS3D multiple protein sequences alignment program was used to align the deduced amino acid sequences of four NySPLs. Secondary structure elements are denoted using blue cylinders (α -helices) and green arrows (β -strands). Identical residues are highlighted in red. Two highly conserved sequences "PSPDW" and "DAGTD" in the FS domain are marked using asterisks. A putative N-glycosylation site is indicated using a box. Numbers represent amino acid positions from the first methionine residue. (**B**) Sequence alignment of fasciclin domain-containing proteins (NyFALs) identified in *Neopyropia yezoensis*. The PROMALS3D multiple protein sequences alignment program was used to align deduced amino acid sequences of fasciclin domains in eight NyFALs. Characteristics of fasciclin H1 and H2 conserved domains are indicated below the alignment. The conserved residue Asp, proposed to be involved in cell adhesion, is marked using an asterisk, and YH motifs are indicated using green arrows.

Table 2. Characteristics of NyFAL proteins identified in Neopyropia yezoensis genome.

Name	Contig ID	Size (aa)	pI	Mw (KDa)	Predicted Location
NyFAL1	contig_12094_g2891	414	8.59	43.5	extracellular
NyFAL2	contig_12570_g3010	1124	4.06	114.15	membrane
NyFAL3	contig_15884_g3808	204	5.37	20.89	cytoplasmic
NyFAL4	contig_23059_g5692	611	11.25	61.98	membrane
NyFAL5	contig_29738_g7294	336	8.98	34.6	mitochondria
NyFAL6	contig_33001_g7988	266	8.55	27.48	endoplasmic reticulum
NyFAL7	contig_38449_g8982	442	5.01	44.87	mitochondria
NyFAL8	contig_39761_g9180	406	4.54	40.53	extracellular

aa-amino acid; pI-isoelectric point; Mw-Molecular weight.

The length of the identified NyFAL proteins ranged from 204 to 1124 amino acids, and predicted pI values ranged from 4.06 to 11.25. NyFAL3, 5, 6, and 8 contained a single fasciclin domain, whereas NyFAL1, 4, and 7 had two fasciclin domains. In contrast, NyFAL2 comprised five fasciclin domains. BLAST analysis showed that the protein sequences of NyFAS exhibited 42–89% identities with fasciclin domain-containing proteins from P. *umbilicalis*. Multiple sequence alignment results showed that the FAS1 domain in NyFALs had conserved H1 and H2 motifs and the YH motif between the H1 and H2 regions (Figure 2B). In addition, seven NyFALs (except for NyFAL5) had conserved aspartic acid residues near the H2 region of the FAS1 domain, similar to those found in human, mice, *Drosophila*, and sea urchins, which are proposed to be essential for cell adhesion activity [51]. It has previously been reported that FAS1 domain-containing proteins from higher plants belong to arabinogalactan proteins (AGPs), named fasciclin-like AGPs (FLAs) [50]. Classical AGPs contain at least 50% PAST (P, proline; A, alanine; S, serine; T, threonine), but FLAs contain less than 35% PAST [52,53]. Among the NyFALs examined in this study, NyFAL4 had a pro-rich region located between two FAS1 domains and contained 64.5% PAST (Pro: 47.6%, Ala: 9.2%, Ser: 3.3%, Thr: 4.2%).

3.3. Expression Profiles of NySPL Genes at Different Developmental Stages

mRNA transcripts of *NySPL* genes were examined at different developmental stages (Figure 3). Expression analysis showed that the transcripts of *NySPL1*–3 genes were highly accumulated in mature gametophytes that formed the spermatia and *NySPL4* had a relatively low expression in sporophytes.

Our previous research revealed that the exogenous application of ACC, a precursor of the phytohormone ethylene, induced gametogenesis in *N. yezoensis* [39,54,55]. Therefore, the expression patterns of *NySPL* genes were examined in response to ACC (Figure 4). The expression of *NySPL1*, 3, and 4 remarkably increased by 35.6 to 91.0-fold after 1 day of ACC treatment, and this increase immediately decreased 7 days after treatment. In contrast, the expression of *NySPL2* increased after 1 day and gradually decreased later. In contrast to sexual reproduction, no remarkable increase in the expression of all *NySPL* genes was observed in gametophytes that formed archeosporangia (Figure S2).

3.4. Expression Profiles of NyFAL Genes at Different Developmental Stages

The amount of *NyFAL1* and *NyFAL4* mRNA was large in sporophytes (272.0-fold to 446.7-fold at vegetative sporophytes, respectively). In contrast, *NyFAL2*, *3*, *5*, *6*, and 7 were highly expressed in gametophytes, especially at the vegetative stage (except for *NyFAL7*) (Figure 5). Results showed that *NyFAL8* was highly expressed in mature gametophytes. The exogenous application of ACC increased *NyFAL8* expression levels by 2.0-fold after 3 days (data not shown). Similarly to the results obtained with *NySPLs*, none of the *NyFALs* remarkable increased transcripts in gametophytes that formed the archeosporangia (Figure S3).



Figure 3. Relative expression levels of *NySPLs* genes in *Neopyropia yezoensis* at different developmental stages. RNA samples were prepared from vegetative gametophytes, which formed vegetative cells only (VGA). Mature gametophytes formed sexual cells (MGA). In contrast, vegetative sporophytes formed vegetative cells only (VSP), whereas mature sporophytes formed conchosporangia (MSP). Expression levels were assessed using the *NyElf1* gene for normalization. Data are presented as means \pm standard deviations (*n* = 3).



Figure 4. Relative expression levels of *NySPLs* genes in *Neopyropia yezoensis* during sexual reproduction in response to 1-aminocylopropane-1-carboxylic acid (ACC). RNA samples were prepared from gametophytes treated with 1-aminocylopropane-1-carboxylic acid (ACC) for 1, 3, or 7 days. Expression levels were assessed using *NyElf1* for normalization. Results were then presented as relative expression and compared with that in non-treated gametophytes (0 day). Data are presented as means \pm standard deviations (*n* = 3).



Figure 5. Relative expression levels of *NyFALs* genes in *Neopyropia yezoensis* at different developmental stages. RNA samples were prepared from vegetative gametophytes, which formed vegetative cells only (VGA). Mature gametophytes formed sexual cells (MGA), whereas vegetative sporophytes formed vegetative cells only (VSP). In contrast, mature sporophytes formed conchosporangia (MSP). Expression levels were assessed using the *NyElf1* gene for normalization. Data are presented as means \pm standard deviations (*n* = 3).

4. Discussion

ECM serves as not only a physical scaffold to cells but also a dynamic structure remodeled by physiological cell conditions. An increasing body of evidence indicates that ECM proteins are involved in the regulation of cell proliferation, migration, adhesion, and differentiation through cell–cell communication and signal transduction in animals and land plants [2,56]. Previous studies showed the different compositions of ECM polysaccharides between gametophyte and sporophyte/vegetative and reproductive stages in Bangiophyceae [10–13]. However, little is known about the role of ECM proteins associated with generation-specific (gametophyte and sporophyte) and reproduction-specific ECM architecture in Bangiophyceae species. In this study, we identified homologous genes encoding ECM proteins such as F-spondin and fasciclin that are known to interact with integrins. The accumulation of knowledge of NySPLs and NyFALs are valuable for understanding roles of ECM systems without integrin family as ECM receptors. Structural studies have revealed that the FS domain from animals exhibits a homology structure similar to that of the C2 domain, which is a membrane-targeting domain involved in signal transduction or membrane trafficking in a Ca²⁺-dependent manner [20,49,57]. The FS domain of F-spondin from humans was found to be a solvent-accessible Ca²⁺-binding site, formed from D325, D354, and D358. Therefore, these results suggest that the FS domain is responsible for part of the Ca²⁺-dependent membrane targeting of F-spondin [20]. NySPLs have similar secondary structures to that of FS domains from animals as well as three conserved aspartic acid residues that serve as Ca²⁺-binding sites (Figure 2). In addition, qRT-PCR analysis showed that NySPLs transcripts increased in gametophytes treated with ACC that is an inducer of the gametogenesis. Thus, NySPLs may function as intercellular adhesion and signal transduction for sexual reproduction in a Ca²⁺-dependent manner.

The spondin family from animals, including F-spondin and Mindin, consists of FS domain and TSR domain [17,58]. On the other hand, the results of our study revealed that all four NySPLs consisted of about 200 amino acids with FS domains, but they lacked a TSR domain that promotes cell adhesion in various cell types [21]. In addition to NySPLs, we found FS domain-containing proteins without TSR in other organisms, such as corals. Although research on F-spondins and mindins that have a TSR domain as well as an FS domain has been advanced, functional analysis of spondins lacking a TSR domain, including NySPLs, is important for understanding the evolution of cell adhesion molecules.

In higher plants, FAS1 domain proteins, which are categorized as a group of HRGPs, have a plethora of biological roles including the maintenance of cell wall architecture and regulation of development [29–34]. Furthermore, a two-FAS1 domain-containing protein obtained from the colonial green alga Volvox carteri, called Algal-CAM, influences the formation of intercellular contacts during early embryogenesis [59]. A study showed that Algal-CAM, in addition to these two FAS1 domains, contained Ser-Pro rich region, with Pro residues that were likely hydroxylated [59]. These findings indicated that the FAS1 domain from green algae and higher plants was closely related to HRGPs, which are important plant cell wall components [28]. Among NyFALs examined in this study, NyFAL4 contains a Pro-rich region between two FAS1 domains, and their transcripts were highly accumulated in the sporophyte stage (Figure 5). The sporophyte of Bangiophyceae has a different cell wall composition (main cell wall polymer is cellulose), morphology (filamentous), and growth behavior (tip growth) compared to the gametophyte [10,60]. Thus, the preferential expression of NyFAL4 in the sporophyte suggests that NyFAL4 may play an important role in cell wall architecture for sporophyte-specific growth and development. In contrast to NyFAL4, NyFAL2, 3, 5, 6, and 7 were highly expressed in gametophytes, which implicates their involvement in development at the gametophyte stage, for example, maintenance of blade shape.

Previous reports showed that FAS1 domain protein from *Arabidopsis*, AtFla4, localized to the developing mucilage pocket of seed coat epidermal cells, and AtFla10 and AtFla17 were identified in a proteome of mature mucilage [61]. Among *NyFALs*, *NyFAL8* transcripts were highly accumulated in mature gametophytes that formed sexual cells (Figure 5). During sexual reproduction in Bangiophyceae species, the mucilage is synthesized in sexual cells, which is responsible for attachment to male and female gametes and the protection of the spores until cell wall deposition [12,13,62]. These findings imply that NyFAL8 may be involved in mature mucilage during *N. yezoensis* reproduction.

N-linked protein glycosylation modulates ECM protein conformation and activity, thereby affecting cell–ECM interactions [63]. In this study, putative N-glycosylation sites were detected within all four NySPL and four NyFAL proteins. Glycosylation reactions are catalyzed by glycosyltransferases, which add sugar chains to various complex carbo-hydrates [64]. Our previous study showed that the transcripts of the *GT14* gene encoding a homolog of N-acetylglucosaminyltransferase, which can transfer N-acetylglucosamine (GlcNAc) to an acceptor substrate, were upregulated in *N. yezoensis* gametophytes that induced sexual reproduction in response to treatment with ACC [65]. In the present study,

we also found that expression levels of *NySPLs* increased after ACC treatment (Figure 4). Thus, N-glycan structures of NySPLs during sexual reproduction should be investigated in the future to elucidate the role of N-linked protein glycosylation in ECM protein activity.

5. Conclusions

N. yezoensis is proposed to be absent in the integrin system, but possesses integrinrelated ECM components, such as spondin domain-containing and fasciclin domaincontaining proteins. Bioinformatics and expressions analysis imply that NySPLs may play an important role in intercellular adhesion and signal transduction for sexual reproduction in a Ca²⁺-dependent manner. NyFALs had conserved aspartic acid residues near the H2 region of the FAS1 domain, which are proposed to be essential for cell adhesion activity in animals. Among NyFALs, NyFAL4 had a pro-rich region located between two FAS1 domains and exhibited sporophyte-preferential expression, suggesting that NyFAL4 may involve in cell wall architecture for filamentous morphogenesis and tip growth at the sporophyte stage. In addition to the functional analysis of NySPLs and NyFLAs, the identification of membrane receptors or other ECM proteins that interact with NyS-PLs and NyFLAs can help us better understand not only the role of ECM remodeling in Bangiophyceae life cycle but also its ancient cell adhesion mechanisms.

Supplementary Materials: The following supporting information can be downloaded at the following website: https://www.mdpi.com/article/10.3390/phycology2010003/s1, Figure S1: Schematic representations of the conserved domains of NySPLs and NyFALs. Domains were determined by searching NySPL and NyFAL sequences in ScanProsite. FS; F-spondin domain, FAS1; Fasciclin 1 domain. The position of the putative N-glycosylation site is indicated using a triangle. Numbers correspond to amino acid positions from the first methionine residue; Figure S2: Relative expression levels of NySPLs genes in Neopyropia yezoensis during asexual reproduction. RNA samples were prepared from gametophytes treated with Hsp90-inhibiting drug, radicicol (RAD), or dimethyl sulfoxide (DMSO, control) for 7 days. Expression levels were assessed using NyElf1 for normalization. Results were then presented as relative expression and compared with that in non-treated gametophytes (0 day). Data are presented as means \pm standard deviations (n = 3); Figure S3: Relative expression levels of NyFALs genes in Neopyropia yezoensis during asexual reproduction. RNA samples were prepared from gametophytes treated with Hsp90-inhibiting drug, radicicol (RAD), or dimethyl sulfoxide (DMSO, control) for 7 days. Expression levels were assessed using NyElf1 for normalization. Results were then presented as relative expression and compared with that in non-treated gametophytes (0 day). Data are presented as means \pm standard deviations (n = 3); Table S1: The list of primers used for gene expression analysis by quantitative Real-Time PCR.

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