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1 **Running head**

2 Proteomics of *P. patens* protonemata subjected to OPDA

3 **Title**

4 Proteomics of *Physcomitrella patens* protonemata subjected to treatment with 12-oxo-
5 phytodienoic acid

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15 **Abbreviations**

16 AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; LC-MS/MS, liquid
17 chromatography tandem mass spectroscopy; LOX, lipoxygenase; OPDA, 12-oxo-phytodienoic

1 acid; PEPC, phosphoenolpyruvate carboxykinase; PGM, phosphoglucomutase; RES, reactive
2 electrophile species;

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1 **Abstract**

2 12-Oxo-phytodienoic acid (OPDA) is induced by mechanical wounding and suppresses the
3 growth of *Physcomitrella patens*; OPDA is considered a signal compound in this moss species. In
4 this study, a proteomic analysis of *P. patens* protonemata treated with OPDA was performed. The
5 abundance levels of 41 proteins were significantly altered by OPDA, with decreased levels for 40
6 proteins. The proteins for which abundance decreased in response to OPDA at the protonema
7 developmental stage were mainly involved in the metabolism of proteins and carbohydrates. The
8 effects of inhibition on protein abundance are likely a major physiological function of OPDA in *P.*
9 *patens*. OPDA also suppressed the expression of histones at the protein level and gene
10 transcription level. Suppression of histone expression might be an OPDA-specific function in *P.*
11 *patens* protonemata. In *P. patens*, a subset of the physiological responses caused by OPDA is
12 shown to differ between protonema and gametophore developmental stages.

13

14 *Keywords*

15 12-Oxo-phytodienoic acid, *Physcomitrella patens*, Proteomic analysis, Protonema, Histones.

16

17 **Introduction**

18 The jasmonates (i.e., jasmonic acid (JA)-related compounds) are synthesized from the fatty
19 acid of the octadecanoid pathway. Jasmonates modulate the expression of numerous genes and

1 mediate responses to various forms of abiotic and biotic stresses.¹⁻⁴⁾ 12-Oxo-phytodienoic acid
2 (OPDA) is a precursor of JA, a plant growth regulator, and plays pivotal roles in plants.⁵⁾ All of
3 the genes encoding JA biosynthesis enzymes have been cloned from several plant species. The
4 corresponding enzymes, including different isoforms, have been characterized.⁶⁾ Previous reports
5 have described the cloning of genes encoding LOX, AOS and AOC in the octadecanoid pathway
6 of *P. patens*; the products of these genes have been shown to have enzymatic activities similar to
7 those in flowering plants.⁷⁻¹⁰⁾ In flowering plants, JA stimulates the expression of a wide variety
8 of genes in response to stress.¹¹⁾ OPDA itself triggers the expression of a set of genes and plays
9 roles in the response to wounding and the regulation of seed dormancy and germination in
10 *Arabidopsis*.¹²⁾ Moreover, OPDA has been reported to play an important role in embryo
11 development and seed germination.^{13, 14)}

12 Bryophytes, which include liverworts, hornworts, and mosses, are considered to be some of
13 first land plants. It is important to elucidate the physiology of bryophytes to understand plant
14 evolutionary processes. Among the bryophytes, *P. patens* is the model moss species. Protein and
15 gene databases and facile gene transformation methods have already been established for *P.*
16 *patens*.¹⁵⁾ The life cycle of *P. patens* is characterized by the following two generations: a haploid
17 gametophyte and diploid sporophyte. A spore develops into a filamentous structure known as
18 protonema. Protonemata can differentiate into gametophores; gametophores are more complex,
19 containing leaf-like structures, rhizoids and sexual organs. As protonemata are distinct from
20 gametophores, gene expression is altered. Therefore, the protein abundance profile in the
21 protonema stage is considered different from that in the gametophore stage.¹⁶⁾

1 Plant hormones regulate a wide variety of physiological events in plants. Studies have
2 demonstrated that plant hormones, such as auxin, cytokinins, and abscisic acid, also control
3 physiological responses in *P. patens*. In *P. patens*, the first half of the octadecanoid pathway
4 exists; however, JA is not synthesized.¹⁰⁾ OPDA inhibits the growth of *P. patens*.¹⁷⁾ Moreover,
5 OPDA was induced when wounding occurs in *P. patens*.¹⁸⁾ Thus, OPDA may be an important
6 oxylin and act as a signaling molecule in *P. patens*.

7 A proteomic analysis of *P. patens* gametophores treated with OPDA was conducted to
8 investigate the function of OPDA.¹⁹⁾ A previous report revealed that OPDA treatment of
9 gametophores resulted in the differential accumulation of several proteins, most of which
10 decreased. As the expression of genes and proteins is altered when differentiation from
11 protonema to gametophore occurs,¹⁶⁾ we are interested in whether the effects of OPDA on protein
12 abundance are different at these two stages of development.

13 In this study, a proteomic analysis of *P. patens* protonemata treated with OPDA was
14 conducted. These data were compared with proteomic data from *P. patens* gametophores. Our
15 findings suggest that OPDA mainly inhibits protein metabolism, which might retard the growth of
16 *P. patens*.

17

18 **Materials and Methods**

19 *Plant growth conditions and treatment*

1 The wild-type strain of *P. patens* subsp. *patens* was used in this study.²⁰⁾ *P. patens* was grown
2 on 20 mL of BCDATG agar medium in a 9-cm petri dish under continuous white fluorescent light
3 at 25 °C.¹⁹⁾ For the microscopic analysis of protonema growth, protonemata grown on the agar
4 plate for 4 days were cut into small pieces using a homogenizer (Polytron PT-10, Kinematica
5 AG, Luzern, Switzerland). After being transferred onto agar supplemented with 5 µM OPDA,
6 10 µM OPDA or 50 µM OPDA, protonemata were incubated for 4 days, after which the
7 phenotypes were observed with a microscope (BZ-9000 fluorescence microscope, Keyence,
8 Osaka, Japan). (+)-*cis*-OPDA synthesis was conducted according to the method described by
9 Kajiwara et al.²¹⁾

10

11 *Analysis of OPDA concentration*

12 *P. patens* was grown on BCDATG agar medium for 5 days, and the concentration of
13 endogenous OPDA was analyzed. Protonemata (approximately 200 mg) were frozen in liquid
14 nitrogen and extracted with 10 mL of ethanol. The OPDA analysis was performed as described by
15 Yamamoto et al.²²⁾ For mechanical stress, the protonemata (approximately 200 mg) were soaked
16 in 2 mL of water and subsequently treated with ultrasonication (Sonifier250, Branson, USA). The
17 samples were directly extracted with 10 mL of ethanol.

18

19 *Protein extraction*

P. patens protonemata were grown on BCDATG agar plates for 5 days and then treated with 10 μ M OPDA (1 ml of an OPDA solution was sprayed directly onto *P. patens* tissues). *P. patens* protonemata were grown under continuous white fluorescent light at 25°C for 24 h. A portion (approximately 2.5 g) of samples (wet *P. patens* tissue) was ground into powder in liquid nitrogen using a mortar and pestle. The powder was transferred into a solution of 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone and mixed. The suspension was sonicated for 5 min and then incubated for 45 min at -20 °C. After this incubation, the suspension was centrifuged at 9,000 *g* for 20 min at 4 °C. The resulting supernatant was discarded, and the pellet was washed three times with 3 mL of acetone containing 0.07% 2-mercaptoethanol. The final pellet was dried using a vacuum pump. The pellet was resuspended by vortexing for 1 h at 25°C in 10 mL of lysis buffer consisting of 100 mM Tris-HCl (pH 8.5), 2% SDS, and 50 mM dithiothreitol (DTT). The suspension was then centrifuged at 20,000 *g* for 20 min at 25°C. The resulting supernatant was collected as the total protein solution. The concentration of the protein solution was measured using the Lorry method.²³⁾

Digestion of proteins

For the in-solution digestion, 100 μ g of protein was subjected to chloroform/methanol extraction.²⁴⁾ The resulting pellet was resuspended with 50 mM NH_4HCO_3 . The solution was reduced with 50 mM DTT and then alkylated with 50 mM iodoacetamide. Proteins were digested using trypsin and lysyl endopeptidase at a 1:100 enzyme/protein ratio at 37°C for 16 h.

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2 *Nanoliquid chromatography-tandem MS analysis*

3 Peptide separation and detection were performed using nanoliquid chromatography tandem
4 mass spectroscopy (nanoLC-MS/MS), which is an Ultimate 3000 nanoLC (Thermo Fisher
5 Scientific, San Jose, CA, USA) and an LTQ Orbitrap mass spectrometer (Thermo Fisher
6 Scientific). The system was operated in data-dependent acquisition mode with the installed
7 XCalibur software (ver. 2.0.7, Thermo Fisher Scientific). The peptides were loaded onto a C18
8 PepMap trap column (300 μ m ID $\times$ 5 mm, Thermo Fisher Scientific). The peptides were eluted
9 from the trap column, and separation and spraying were performed using 0.1% formic acid in
10 acetonitrile at a flow rate of 200 nL/min on a C18 Tip column (75 μ m ID $\times$ 120 mm, Nikkyo
11 Technos, Tokyo, Japan) with a spray voltage of 1.5 kV. Elution was performed with a linear
12 acetonitrile gradient (5-25% in 120 min) in 0.1% formic acid. Full-scan mass spectra were
13 acquired in the Orbitrap over 400-1,500 m/z with a resolution of 30,000. A lock mass function
14 was used to obtain high mass accuracy.²⁵⁾ The top ten most intense precursor ions were selected
15 for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35%.
16 Dynamic exclusion was employed within 90 s to prevent repetitive peptide selection.²⁶⁾

17

18 *Protein identification using MASCOT*

19 Acquired MS/MS spectra were subjected to protein identification using MASCOT software
20 (ver. 2.4.1, Matrix Science, London, UK) and the *P. patens* database (38,480 protein sequences)

1 via Proteome Discoverer (ver. 1.4.0.288, Thermo Fisher Scientific). The *P. patens* database was
2 obtained from Phytozome Database (ver. 9.1, <http://www.phytozome.net/>). The parameters used
3 in the Mascot searches were as follows: the carbamidomethylation of cysteine was set as a fixed
4 modification; the oxidation of methionine was set as a variable modification; trypsin was
5 specified as the proteolytic enzyme; and one missed cleavage was allowed. The peptide mass
6 tolerance was set at 10 ppm. The fragment mass tolerance was set at 0.8 Da, and the peptide
7 charge was set at +2, +3, and +4. An automatic decoy database search was performed within the
8 search. Mascot results were filtered using the percolator function in Proteome Discoverer to
9 improve the accuracy and sensitivity of peptide identification.²⁷⁾

11 *Analysis of differential protein abundance using the acquired MS data*

12 For differential analyses, the commercial label-free quantification package SIEVE (ver. 2.1,
13 Thermo Fisher Scientific) was used to compare the relative abundance of peptides and proteins
14 between the control and experimental groups. The chromatographic peaks detected by MS were
15 aligned, and the peptide peaks were detected as frame using the following settings: the frame time
16 width was 5.0 min; the frame m/z width was 10 ppm; and frames were produced on all parent ions
17 subjected to MS/MS scanning. The frames with MS/MS scans were matched to the imported
18 MASCOT results. The MASCOT results were imported with following settings: a minimum
19 peptide ion score of 13 and a maximum percolator q-value of 0.01. The total ion current was used
20 as a normalization factor for the differential analysis.

1

2 *mRNA expression analysis*

3 Total RNA was extracted from protonemata with the inunuPREP Plant RNA Kit (Analytik
4 Jena Life Science, Jena, Germany). Two microliters of RNA was used for first-strand cDNA
5 synthesis with M-MLV reverse transcriptase (Invitrogen, Carlsbad, California, USA) according to
6 the manufacturer's instructions. Gene-specific primers were designed according to expressed
7 sequence tag data available for *P. patens*. The sequences of the primers are listed in Supplemental
8 Table 1. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was
9 performed following the manufacturer's protocols (SYBR Premix Ex Taq II, Takara Bio Inc.,
10 Shiga, Japan). All RT-PCR reactions were run with three biological replicates on a Thermal
11 Cycler Dice TP800 real-time PCR system (software ver. 3.00D, Takara, Japan). To normalize
12 gene expression, *actin 5* was used as an internal standard set to 1.0.²⁸⁾

13

14 **Results**

15 *Protein identification and functional categories of differentially expressed proteins*

16 OPDA was shown to suppress the colony growth and rhizoid length of *P. patens* under a
17 concentration of 10 μ M,¹⁷⁾ and mechanical stress transiently stimulates OPDA accumulation.¹⁹⁾
18 The morphology of *P. patens* varies over the course of the life cycle. The protonema and
19 gametophore developmental stages are quite different.²⁹⁾ We examined the effects of OPDA on
20 the growth of *P. patens* protonemata. *P. patens* protonemata were incubated in a medium
21 supplemented with OPDA and were then viewed under a microscope (Fig. 1). OPDA retarded the

1 growth of protonemata of *P. patens* in a concentration-dependent manner. When OPDA
2 concentration was more than 10 μ M, the protonema growth was clearly inhibited by OPDA. In
3 contrast to OPDA, JA did not show significant growth inhibitory effect for *P. patens* protonemata.
4 Analytical data of endogenous OPDA concentration showed that mechanical wounding
5 transiently elevated OPDA concentration in *P. patens* protonema in a similar way with higher
6 plants (Fig. 2). These results strongly suggest OPDA signaling system functions in protonema
7 and gametophore stages in *P. patens*.

8 A previous report demonstrated that OPDA alters the abundance of proteins involved in light-
9 dependent reactions, the octadecanoid pathway, carbon fixation, glycolysis and protein synthetic
10 processes in gametophores.¹⁹⁾ To examine the effects of OPDA on protein abundance for *P.*
11 *patens* protonemata, a proteomic analysis of *P. patens* protonemata treated with 10 μ M OPDA for
12 24 h was performed. The extracted proteins were digested with trypsin and lysyl endopeptidase,
13 and the resulting peptides were analyzed using nanoLC-MS/MS. The protein levels were
14 compared based on the area under the curve of each matched peptide using SIEVE software; the
15 number of proteins that were matched with more than two peptides was 2662. A subsequent
16 comparative analysis of OPDA-treated protonemata and untreated protonemata revealed that 41
17 proteins were differentially changed with fold changes of at least 1.5 ($p < 0.05$) (Table 1). The
18 abundance of 40 proteins decreased; only one protein increased in abundance due to OPDA
19 treatment in protonemata.

20 Based on their biological properties, these differentially changed proteins were grouped into
21 the following six categories: defense, energy and carbohydrate metabolism, photosynthesis,

1 protein metabolism (proteins synthesis, folding and degradation), others and unknown (Fig. 2).
2 Twenty-one differentially changed proteins are involved in protein synthesis. One protein is
3 involved in defense, and five proteins are involved in carbohydrate and energy metabolism.

4

5 *Proteomic comparison of gametophores and protonemata*

6 We compared the proteome data of gametophores¹⁹⁾ and protonemata to analyze the function
7 of OPDA in *P. patens*. As shown in Fig. 2, 82 proteins were differentially expressed in response
8 to OPDA treatment in gametophores (threshold level: 2.5-fold change). The amounts of 41
9 proteins were differentially altered in protonemata (threshold level: 1.5-fold change). The
10 abundance of most proteins decreased following OPDA treatment in both developmental stages.

11 The proteins that decreased in abundance in protonemata and gametophores accounted for
12 majority of the proteins that were differently accumulated proteins due to OPDA treatment.
13 Proteins involved in protein metabolism, energy and carbohydrate metabolism accounted for
14 approximately 65 % and 73 % of the proteins that decreased in abundance due to OPDA
15 treatment in protonemata and gametophores, respectively. These results suggest that the primary
16 mode of action of OPDA is the repression of protein metabolism and energy consumption
17 processes at both developmental stages. Proteins involved in photosynthesis accounted for 100 %
18 and 83 % of the proteins that increased abundance due to OPDA treatment in protonemata (one
19 protein) and gametophores, respectively. These results suggest that OPDA would act on

1 photosynthesis in both gametophores and protonemata. The abundance suppression of several
2 histones was notable in the protonema of *P. patens* protonemata treated with OPDA.

3

4 *Proteins with increased abundance due to OPDA treatment of protonemata*

5 The abundance of only one protein, oxygen-evolving enhancer protein 2 (OEE2), was
6 increased by OPDA in protonemata. This protein is encoded by the nuclear genome. OEE2 is
7 required for high levels of O₂ evolution. Oxygen-evolving enhancer protein (Pp1s61_321V6.1)
8 belongs to the PsbP family, which is required for increased PSII affinity for the water oxidation
9 site of Cl⁻ and provides the conditions required for high affinity binding to Ca²⁺.³⁰⁾ Both PsbP and
10 PsbQ are necessary regulators of the biogenesis of optically active PSII. The oxygen-evolving
11 complex (OEC) is responsible for catalyzing the splitting of water to O₂ and H⁺. In flowering
12 plants, such as *Arabidopsis*, OPDA plays an important role in response to biotic and abiotic
13 stresses.⁵⁾ OPDA is accumulated in *P. patens* gametophores due to wounding. OPDA
14 accumulation results in the increased protein abundance in the gametophore stage, including PsbC,
15 psbD, and psbE and allene oxide cyclase (AOC).¹⁹⁾ Accordingly OPDA is likely synthesized to
16 stimulate light-dependent reactions in response to mechanical stress.

17

18 *Decreased proteins involved in carbohydrate metabolism and energy production*

19 OPDA treatment resulted in the decreased abundance of enzymes involved in carbohydrate
20 metabolism, such as phosphoenolpyruvate carboxykinase, phosphoglucomutase,

phosphoglycerate kinase and pyruvate kinase. Phosphoenolpyruvate carboxykinase (PEPC) is the bottleneck enzyme for gluconeogenesis, which catalyzes the addition of bicarbonate to phosphoenolpyruvate to form oxaloacetate and inorganic phosphate. PEPC plays a crucial role in modulating the balance of carbon metabolism in *Arabidopsis*.³¹⁾ Phosphoglucosmutase (PGM) catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate; the enzyme exists in both plastidial and cytosolic isoforms. The plastidial isoform is essential for transitory starch synthesis in the chloroplasts of leaves, whereas the cytosolic counterpart is essential for glucose phosphate partitioning and the synthesis of sucrose and cell wall components. A lack of PGM (both plastidial and cytosolic isoforms) activities in *Arabidopsis* resulted in dwarfed growth, premature death, and an inability to develop a functional inflorescence.³²⁾ The synthesis of the enzymes phosphoglycerate kinase and pyruvate kinase was suppressed by OPDA; these enzymes are involved in carbon fixation (Calvin cycle) and energy production. Phosphoglycerate kinase is an essential enzyme in the Calvin cycle; it catalyzes the phosphorylation of 3-phosphoglycerate with ATP, which is produced in the light-dependent stage.³³⁾ Pyruvate kinase catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP to yield pyruvate and ATP.³⁴⁾

Proteins with decreased abundance involved in protein metabolism

OPDA suppressed the abundance of ribosomal proteins, transcriptional initiator and translational inhibitor proteins; these proteins are associated with RNA. Of the 40 proteins with suppressed abundance, over half were involved in protein metabolism (Table 1, Fig. 2C). OPDA

1 was shown to down-regulate protein synthesis by suppressing transcription and translation
2 activity. Among the OPDA-repressed proteins involved in protein metabolism, ribosomal proteins
3 were mainly identified in protonemata. The repression of ribosomal protein biosynthesis
4 significantly affects various physiological phenomena in cells. Translational inhibitor proteins
5 (endoribonuclease L-PSP) are active on single-stranded mRNA and inhibit protein synthesis by
6 cleaving mRNA.^{35, 36)} When protein synthesis is suppressed in cells, translational inhibitor
7 proteins might be unnecessary. The repression of protein synthesis likely leads to a decreased
8 abundance of translational inhibitor proteins.

9

10 *Six histones were suppressed by OPDA*

11 The abundance of six histones was decreased in *P. patens* protonemata treated with OPDA.
12 To our knowledge, this is the first report that OPDA inhibits histone abundance. These histones
13 all belong to the histone H2A group. Histones are the chief components of chromatin and play an
14 important role in the regulation of gene expression. Histone H2A is important for the packaging
15 of DNA into chromatin; this packaging process is believed to affect gene expression.³⁷⁾ Our
16 results revealed that the abundance of these six histones decreased due to OPDA treatment.
17 Considering the function of histones in gene expression, this finding was worthy of further
18 exploration. We analyzed the expression of these six histone genes at the transcription level by
19 qRT-PCR. As a result, 10 μ M OPDA was shown to down-regulate the mRNA expression of these
20 histone genes (Fig. 3). These results supported the proteomic data in this study. The qRT-PCR

1 data indicate that OPDA regulates the expression of these histone genes at the transcriptional
2 level. It is likely that OPDA affects cell cycle progression at the protonema stage of *P. patens*.

3

4 **Discussion**

5 A comparison of the proteome data from *P. patens* protonemata treated with OPDA with that
6 from *P. patens* gametophores treated with OPDA revealed that greater number of proteins were
7 affected in gametophores than in protonemata. Additionally, the magnitude of the changes in
8 protein abundance evoked by OPDA in gametophores was greater than in protonemata. As
9 protonemata differentiate into gametophores, physiological events become more complex; this
10 may explain why OPDA on protein abundance appear to be more significant in gametophores.
11 Whereas OPDA elevates the abundance of a set of proteins in *Arabidopsis*,¹²⁾ the abundance of
12 most proteins altered by OPDA decreased in *P. patens*. Contrary to the changes in protein
13 accumulation due to OPDA observed in *Arabidopsis*, changes in protein accumulation were
14 restrained in *P. patens*.

15 Changes in the abundance of proteins involved light-dependent reactions were elicited by
16 OPDA in protonemata and gametophores. Accordingly, it was hypothesized that OPDA enhances
17 light-dependent reactions in *P. patens*. Light-dependent reactions provide oxygen, which is
18 connected to the production of reactive electrophile species (RES). RES and lipid peroxidation
19 appear to be advantageous in plant cells when plants are subjected to stress. RES is presumed to
20 induce the expression of genes related to cell survival.³⁸⁾ As light-dependent reactions are induced

1 by OPDA, redox changes that stimulate signaling cascades to induce the nuclear transcription of
2 mediators may occur.³⁹⁾ OPDA-induced proteins in light-dependent reactions seem to play roles
3 in the stress response at both the protonema and gametophore stages of *P. patens*. In *Arabidopsis*,
4 OPDA elevates the accumulation of some proteins involved in photosynthesis; however, the
5 abundance of Rubisco was decreased due to the toxicity of 100 μ M OPDA.³⁹⁾ While a high OPDA
6 concentration might give a harmful effect to plants, OPDA treatment resulted in an induction of
7 the synthesis of photosynthesis-related proteins. Taken together, it is likely that the increased
8 abundance of photosynthesis-related proteins due to OPDA treatment is conserved in land plants.

9 A previous proteomic analysis of *P. patens* gametophores demonstrated that OPDA treatment
10 induced the abundance of proteins encoded by genes in the chloroplast genome; these proteins
11 were involved in light-dependent reactions.¹⁹⁾ In protonemata, no proteins encoded in the
12 chloroplast genome were altered by OPDA treatment. The OPDA-induced accumulation of
13 proteins encoded in the chloroplast genome, which are involved in light-dependent reactions, may
14 be a physiological response of *P. patens* that is specific to gametophores. The abundance of AOC
15 proteins was induced by OPDA in *P. patens* gametophores, indicating the presence of positive
16 feedback regulation on OPDA biosynthesis in *P. patens* gametophores. In contrast, AOC
17 abundance was not increased in *P. patens* protonemata. Protonema stage is an active growing
18 stage in the life cycle of *P. patens*, therefore protonemata grow more rapidly than gametophores.
19 Given that OPDA retards growth in *P. patens*, the positive feedback regulation on OPDA
20 biosynthesis might be suppressed during the protonema stage.

OPDA also reduced the abundance of proteins involved in proteins synthesis and carbohydrate metabolism. More than 50% of the affected proteins were involved in protein metabolism (Table 1, Fig. 2C). OPDA treatment mainly reduced the abundance of ribosomal proteins in protonemata. Ribosomes are the primary apparatus for biological protein synthesis. The functional repression of ribosomes disrupts the generation of new proteins, thereby arresting the cell growth. As the protonema stage is a period of active growth, more so than the gametophore stage, the growth inhibition observed due to OPDA treatment might be the result of decreased ribosomal protein abundance in *P. patens* protonemata. Whereas OPDA decreased the abundance of proteins involved in protein metabolism in both protonemata and gametophores, the abundance of additional proteins involved in protein metabolism and amino acid synthesis was reduced in gametophores. OPDA likely repressed protein synthesis multilaterally in gametophores.

The abundance of proteins related to carbohydrate metabolism was also suppressed by OPDA treatment in *P. patens* protonemata; these proteins were not identified in a previous study of gametophores. Given that OPDA synthesis is triggered by wounding, the inhibitory effects of OPDA on the abundance of proteins involved in carbohydrate metabolism have likely been adapted to adverse environmental conditions.

OPDA treatment decreased the accumulation of histones in *P. patens* protonemata. The decline in histone accumulation might affect DNA replication, as histones are important components of nucleosomes.⁴⁰⁾ Protonemata grow by apical cell division under the influence of cytokinin; buds are derived from three-faced apical cells that differentiate into gametophores, which contain stem- and leaf-like structures.²⁹⁾ Moreover, cell differentiation is relatively slow in

1 the gametophore stage of *P. patens*. Protonemata grow more rapidly compared with
2 gametophores. Therefore, suppression of histone expression due to OPDA treatment may retard
3 growth more severely in protonemata than in gametophores.

4 Genomic analysis revealed that the COI-JAZ system is also present in *P. patens*. However JA
5 does not show any significant effect in *P. patens*. The inability of JA to bind COI1 likely causes
6 little physiological response in *P. patens*. It is likely that OPDA and/or an identified OPDA-
7 related compound binds COI for activation of OPDA signaling in *P. patens*. Alternatively *P.*
8 *patens* might have a unique OPDA signaling system. In either case, considering that OPDA yields
9 physiological effects in land plants, an OPDA signaling system might have been conserved since
10 the emergence of land plants.

11 In conclusion, OPDA results in the decreased abundance of proteins involved in the
12 metabolism of proteins and carbohydrates at the protonema and gametophore developmental
13 stages. The inhibition of protein synthesis is likely one of main physiological functions of OPDA
14 in *P. patens*. This study demonstrated that OPDA suppressed histones expression at both protein
15 level and gene transcription level at the protonema stage. The elucidation of detailed OPDA
16 signaling mechanisms shed light on *P. patens* physiology and plant evolution. Our study
17 advances the knowledge of how OPDA regulates physiology in *P. patens* and increases our
18 understanding of the function of OPDA as a signaling compound in plants.

19

20 **Authors contributions**

1 Study concept and design: Kosaku Takahashi. Acquisition of data: Weifeng Luo, Yohei
2 Nanjo, Setsuko Komatsu, and Kosaku Takahashi. Analysis and interpretation of data: Weifeng
3 Luo, Yohei Nanjo, Setsuko Komatsu, Hideyuki Matsuura, Kosaku Takahashi. Drafting of the
4 manuscript: Weifeng Luo and Kosaku Takahashi. All authors reviewed and approved the final
5 manuscript.

6

7 **Disclosure statement**

8 No potential conflict of interest was reported by the authors.

9

10 **Supplemental material**

11 Supplemental material for this article can be accessed at

12

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4

5 **Figure legends**

6 Fig. 1. Effects of OPDA on protonema prolongation in *P. patens*.

7 Protonemata were grown on BCDATG agar plates with OPDA or JA for 4 days. The control
8 showed protonemata on the agar plate without OPDA and JA. Scale bar shows 200 μ m.

9

10 Fig. 2. Accumulation of OPDA in *P. patens* protonemata after mechanical stress.

11 *P. patens* protonemata were grown on BCDATG agar medium for 4 days, and the concentration
12 of endogenous OPDA was analyzed. After *P. patens* protonemata were subjected to mechanical
13 stress for the indicated times, the protonemata were harvested and the OPDA concentration was
14 analyzed using UPLC-MS/MS. The values are the means $\pm$ SD (n = 4). Student's *t* test, **p* < 0.05,
15 ***p* < 0.01, ****p* < 0.001.

16

17 Fig. 3. Comparison of proteins changed in gametophore and protonema.

18 A: comparison of the global protein response to OPDA; B: comparison of proteins increased by
19 OPDA; C: comparison of proteins decreased by OPDA. PH: photosynthesis; PR: protein

1 metabolism (protein synthesis, folding and degradation); EM: energy and carbohydrate
2 metabolism; DE: defense; UK: unknown. Gametophore data are derived from a paper by Toshima
3 et al.¹⁹⁾

4

5 Fig. 4. Relative expression of histone genes by qRT-PCR.

6 Five-day-old *P. patens* protonemata were treated with 10 μ M OPDA or water for 24 h. The
7 expression level of the internal standard gene *actin 5* is set to 1.0. The data represent means $\pm$ SD
8 (n=3). Asterisks represent significant differences between OPDA-treated plants and control plants
9 (Student's t test, $*p < 0.05$). Histone A: Pp1s117_154V6.1; Histone B: Pp1s219_44V6.1; Histone
10 C: Pp1s376_22V6.1; Histone D: Pp1s46_245V6.1; Histone E: Pp1s72_85V6.1; Histone_F:
11 Pp1s72_86V6.1.

1 Table 1. Proteins identified as responsive to OPDA in *P. patens* protonemata.

Protein ID ¹	Description	Category ²	MP ³	FC ⁴
Pp1s61_321V6.1	oxygen-evolving enhancer protein chloroplast	PH	4	1.60
Pp1s253_38V6.1	40S ribosomal protein S6	PR	3	0.67
Pp1s253_7V6.1	40S ribosomal protein S6	PR	3	0.67
Pp1s311_33V6.1	40S ribosomal protein S6	PR	3	0.67
Pp1s31_322V6.1	40S ribosomal protein S6	PR	3	0.67
Pp1s264_34V6.1	proliferation-associated protein 2g4	PR	2	0.67
Pp1s271_9V6.1	40s ribosomal protein	PR	2	0.66
Pp1s2_233V6.1	60s ribosomal protein l18	PR	2	0.66
Pp1s107_27V6.1	unknown	UK	3	0.66
Pp1s47_196V6.1	apoptosis inhibitor	DE	3	0.65
Pp1s45_11V6.1	60s ribosomal protein l18	PR	2	0.65
Pp1s306_12V6.1	hypothetical PE-PGRS family protein PE_PGRS54 precursor	UK	2	0.64
Pp1s145_142V6.1	phosphoglycerate kinase	EM	2	0.64
Pp1s159_52V6.1	phosphoenolpyruvate carboxykinase	EM	4	0.64
Pp1s60_266V6.1	phosphoenolpyruvate carboxykinase	EM	4	0.64
Pp1s221_62V6.1	formamidopyrimidine-dna glycosylase	Others	5	0.63
Pp1s168_70V6.2	phosphoglucomutase a	EM	7	0.63
Pp1s42_99V6.1	lupus la	PR	2	0.62
Pp1s117_154V6.1	histone h2a	Others	2	0.61
Pp1s219_44V6.1	histone 2	Others	2	0.61
Pp1s376_22V6.1	histone 2	Others	2	0.61
Pp1s46_245V6.1	histone 2	Others	2	0.61
Pp1s72_85V6.1	histone h2a	Others	2	0.61
Pp1s72_86V6.1	histone h2a	Others	2	0.61
Pp1s198_153V6.2	inner membrane protein	Others	3	0.60
Pp1s198_153V6.3	inner membrane protein	Others	3	0.60
Pp1s136_70V6.1	glycine-rich RNA-binding protein	PR	3	0.60
Pp1s311_33V6.2	40S ribosomal protein S6	PR	2	0.59
Pp1s37_67V6.1	translational inhibitor protein	PR	2	0.59
Pp1s37_67V6.2	translational inhibitor protein	PR	2	0.59
Pp1s37_67V6.3	translational inhibitor protein like	PR	2	0.59
Pp1s114_93V6.1	pyruvate kinase	EM	2	0.59
Pp1s247_51V6.1	translation initiation factor	PR	3	0.57
Pp1s97_246V6.1	unknown	UK	5	0.53
Pp1s79_93V6.1	FCAALL.30; lil3 protein	PR	3	0.53
Pp1s165_40V6.1	60s ribosomal protein	PR	3	0.51
Pp1s174_48V6.1	60s ribosomal protein	PR	3	0.51
Pp1s63_162V6.1	60s ribosomal protein	PR	3	0.51
Pp1s80_110V6.1	60s ribosomal protein	PR	3	0.51
Pp1s75_223V6.1	60s ribosomal protein	PR	4	0.51
Pp1s83_99V6.1	plastid-lipid-associated protein 2	PH	2	0.50

2

3 ¹Protein IDs are from Phytozome ver. 9.1 (<http://www.phytozome.net/>).

4 ²Category: DE, defense; EM, energy and carbohydrate metabolism; PH, photosynthesis; PR,
5 protein metabolism (protein synthesis, folding and degradation); UK, unknown. ³MP indicates the
6 number of matched peptides. ⁴FC indicates the fold change between control and 10 μ M OPDA
7 treatment.

Fig. 1. (Luo et al.)

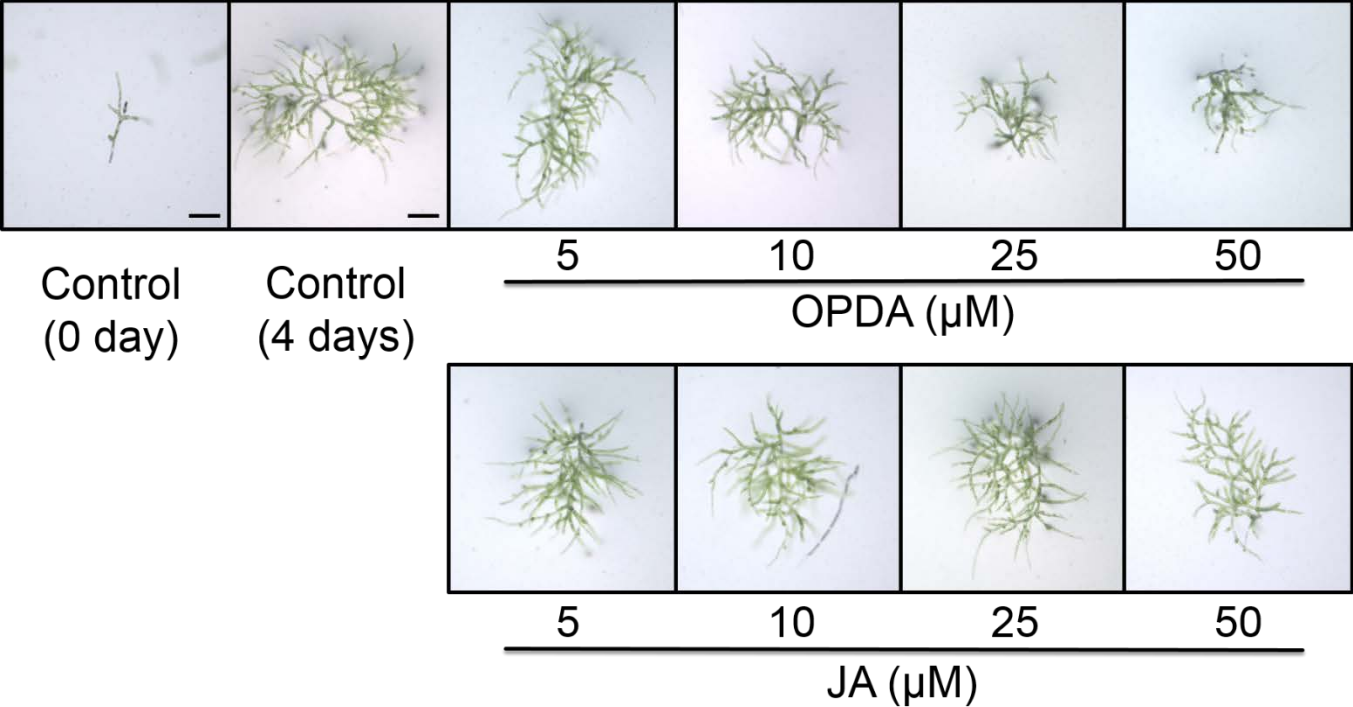


Fig. 2. (Luo et al.)

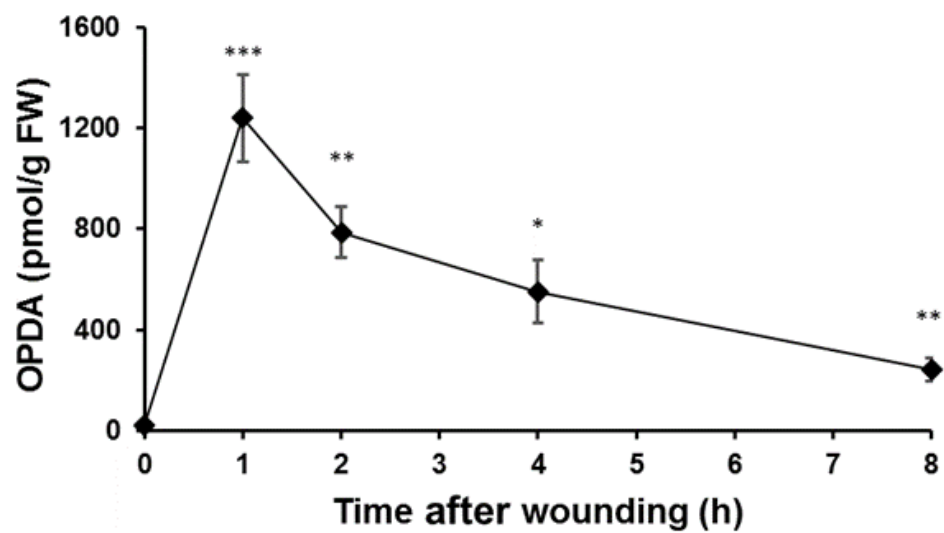


Fig. 3. (Luo et al.)

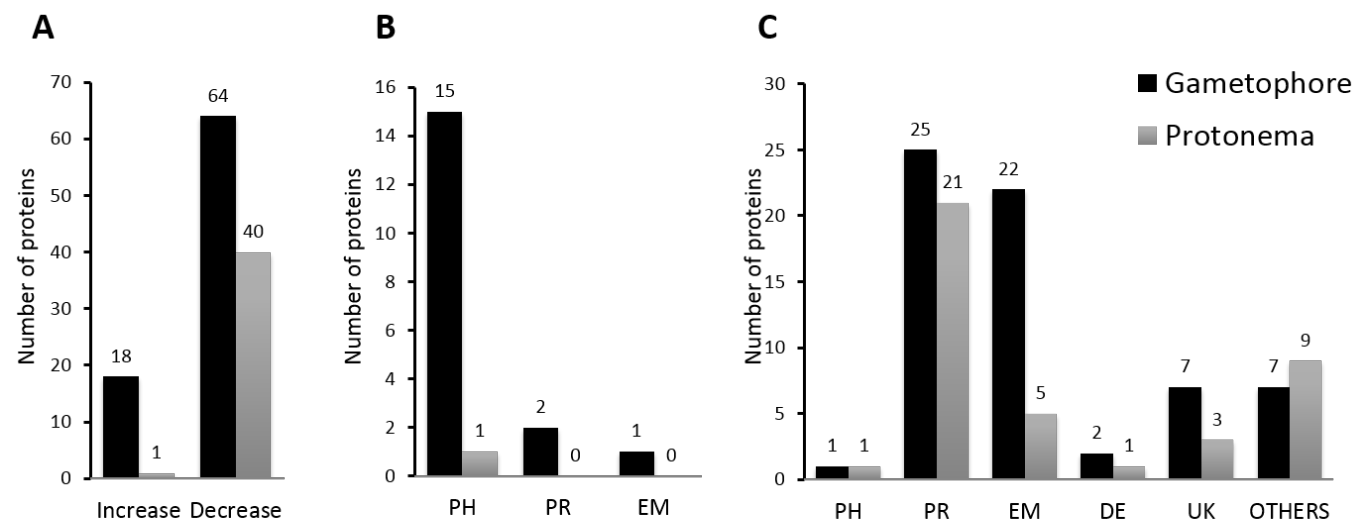
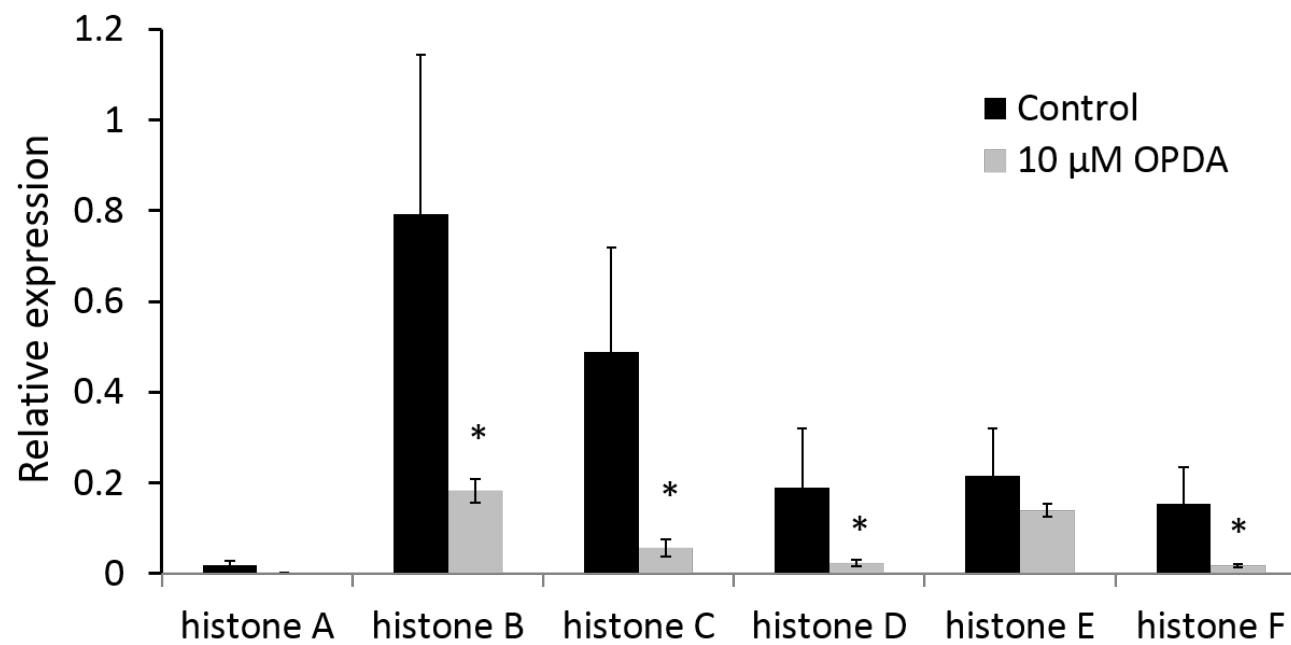
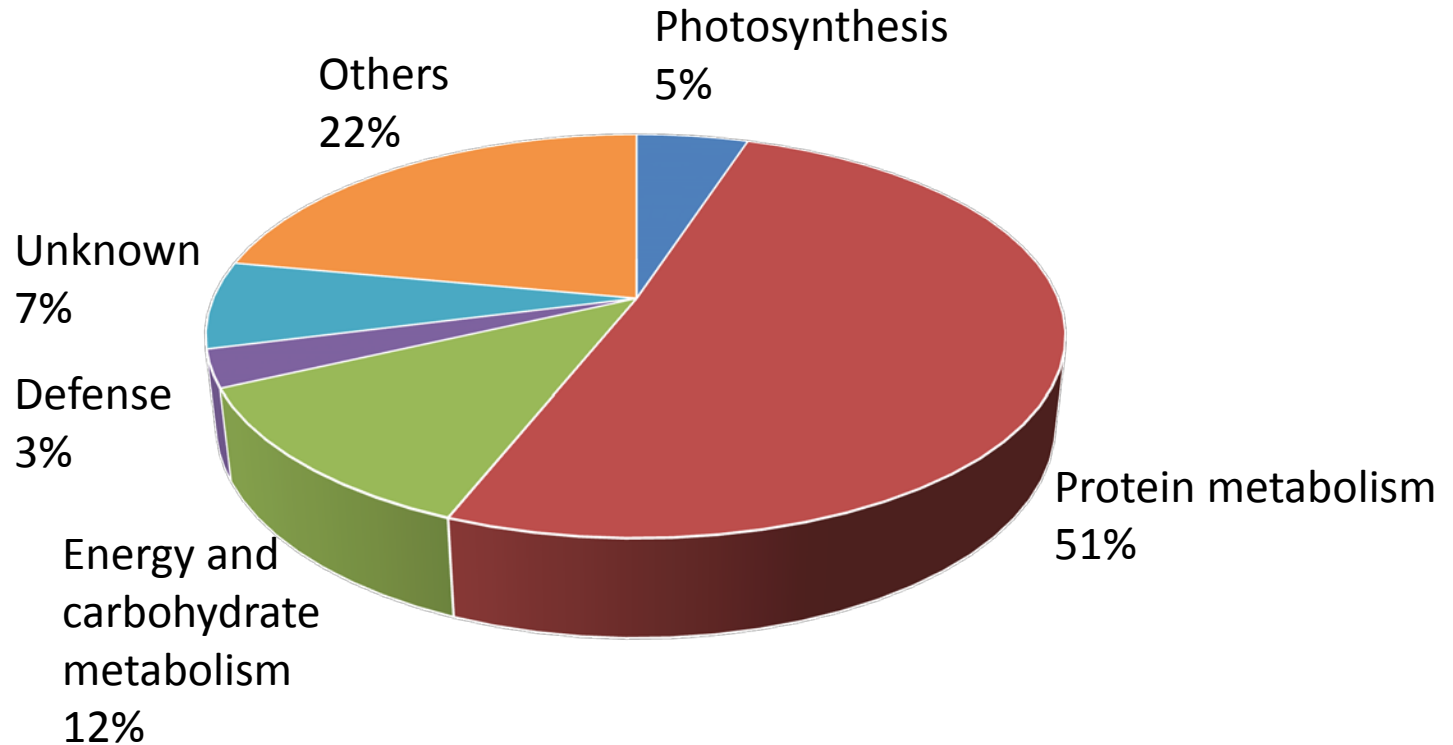


Fig. 4. (Luo et al.)





Classification of OPDA-regulated proteins in
Physcomitrella patens protonema according
to their functions

Supplementary Table 1. List of primers used in qRT-PCR expression analysis.

Primer description	Protein description	Protein ID	Primer sequence
Histone_A_A	Histone h2a	Pp1s117_154V6.1	AGGCGTGCTTCCTAACATTCACAG
Histone_A_S	Histone h2a	Pp1s117_154V6.1	TAAGACAATACGAACGGGCGGTGT
Histone_B_A	Histone h2a	Pp1s219_44V6.1	CATCTCGTGCTGGTCTTCAGTTCC
Histone_B_S	Histone h2a	Pp1s219_44V6.1	CCTTCAGATCCTTACTAGCGTTTCC
Histone_C_A	Histone h2a	Pp1s376_22V6.1	TTAGTTGTGAAGTGCTCGGTTGGT
Histone_C_S	Histone h2a	Pp1s376_22V6.1	GAAGTAGGCTTCTTCTTATCCTTGTTTT
Histone_D_A	Histone h2a	Pp1s46_245V6.1	TCGTTACGCTGAGATCATGTGAGGC
Histone_D_S	Histone h2a	Pp1s46_245V6.1	GAACACCCAGACAGTAGTTAGTTGAAAA
Histone_E_A	Histone h2a	Pp1s72_85V6.1	TGATTGGACCTGCGATCTTGAC
Histone_E_S	Histone h2a	Pp1s72_85V6.1	GGGAGAATGCCTTGGTGCTGAA
Histone_F_A	Histone h2a	Pp1s72_86V6.1	CGTTCGTTTATTGTTGTGATAGGGTTA
Histone_F_S	Histone h2a	Pp1s72_86V6.1	CTCAAGGACCTCAGCAGCCAGA
Ppactin-3U1	Actin		CGGAGAGGAAGTACAGTGTGTGGA
Ppactin-3D1	Actin		ACCAGCCGTTAGAATTGAGCCCAG

The primers for actin are referenced in the following paper: Aoki S, Kato S, Ichikawa K, and Shimizu M. Circadian expression of the *PpLhcb2* gene encoding a major light-harvesting chlorophyll a/b-binding protein in the moss *Physcomitrella patens*. Plant Cell Physiol., 45, 68–76 (2004).