Functions of serine from the phosphorylated pathway on growth, male gametogenesis, and metabolism in Marchantia polymorpha

Masami Hirai (masami.hirai@riken.jp)
RIKEN Center for Sustainable Resource Science  https://orcid.org/0000-0003-0802-6208

Mengyao Wang
RIKEN Center for Sustainable Resource Science

Hiromitsu Tabeta
RIKEN Center for Sustainable Resource Science

Kinuka Ohtaka
Japan Women’s University

Ayuko Kuwahara
RIKEN Center for Sustainable Resource Science

Ryuichi Nishihama
Tokyo University of Science  https://orcid.org/0000-0002-7032-732X

Toshiki Ishikawa
Saitama University  https://orcid.org/0000-0001-8083-6542

Kiminori Toyooka
RIKEN  https://orcid.org/0000-0002-6414-5191

Mayuko Sato
RIKEN  https://orcid.org/0000-0002-9876-5612

Mayumi Wakazaki
RIKEN Center for Sustainable Resource Science

Hiromichi Akashi
RIKEN Center for Sustainable Resource Science

Hiroshi Tsugawa
Tokyo University of Agriculture and Technology  https://orcid.org/0000-0002-2015-3958

Tsubasa Shoji
RIKEN Center for Sustainable Resource Science

Yozo Okazaki
Mie University

Keisuke Yoshida
Tokyo Institute of Technology  https://orcid.org/0000-0001-6542-6254

Ryoichi Sato
Article

Keywords:

Posted Date: April 10th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2663856/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: There is NO Competing Interest.
Functions of serine from the phosphorylated pathway on growth, male gametogenesis, and metabolism in *Marchantia polymorpha*

Mengyao Wang\(^1,2\)#, Hiromitsu Tabeta\(^1,3,4,\)#, Kinuka Ohtaka\(^1,2,5,\)#, Ayuko Kuwahara\(^1\), Ryuichi Nishihama\(^6,7\), Toshiki Ishikawa\(^8\), Kiminori Toyooka\(^1\), Mayuko Sato\(^1\), Mayumi Wakazaki\(^1\), Hiromichi Akashi\(^1\), Hiroshi Tsugawa\(^1,9\), Tsubasa Shoji\(^1\), Okazaki Yozo\(^1,10\), Keisuke Yoshida\(^11\), Ryoichi Sato\(^1\), Ali Ferjani\(^4\), Takayuki Kohchi\(^6\), Masami Yokota Hirai\(^1,2\)*

\(^1\) RIKEN Center for Sustainable Resource Science
\(^2\) Graduate School of Bioagricultural Sciences, Nagoya University
\(^3\) Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo
\(^4\) Department of Biology, Tokyo Gakugei University
\(^5\) Department of Chemical and Biological Sciences, Faculty of Science, Japan Women’s University
\(^6\) Graduate School of Biostudies, Kyoto University
\(^7\) Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science
\(^8\) Graduate School of Science and Engineering, Saitama University
\(^9\) Biotechnology and Life Science, Institute of Engineering, Tokyo University of Agriculture and Technology
\(^10\) Graduate School of Bioresource, Mie University
\(^11\) Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology

#These authors contributed to the work equally and should be regarded as co-second authors.

*Corresponding author:
Masami Yokota Hirai
RIKEN Center for Sustainable Resource Science
Phone: +81-045-503-7040, Fax: +81-045-503-9489
E-mail: masami.hirai@riken.jp
Abstract
Serine is an important precursor of various biomolecules, including lipids. In this study, we
investigated the role of the phosphorylated pathway of serine biosynthesis in a non-vascular
plant *Marchantia polymorpha* by analyzing knockout mutants of *MpPGDH*, a single gene
encoding the first committed enzyme 3-phosphoglycerate dehydrogenase (PGDH), to assess
primary functions of this pathway in relation to those of the other two pathways. Growth
phenotypes of the knockout mutants under different light conditions indicated that serine
supply from the phosphorylated pathway in the dark was crucial for vegetative growth.
Sperm formation required serine from the phosphorylated pathway, while egg formation did
not depend on it. Knockout of *MpPGDH* in the maternal genome disrupted sporophyte
development after fertilization. When the *Mppgdh* mutants were grown in high CO$_2$ where
the photorespiratory glycolate pathway for serine biosynthesis is inhibited, thallus growth
was suppressed and not fully recovered to wild-type level by exogenous serine supplement,
suggesting that serine homeostasis involving both the phosphorylated and glycolate pathways
was essential. Metabolome and lipidome analyses indicated that the phosphorylated pathway
mainly influenced the tricarboxylic acid cycle, the amino acid and nucleotide metabolism,
and lack of serine caused significant perturbation in lipid metabolism. Overall, our results
indicate the importance of serine from the phosphorylated pathway for sperm formation,
sporophyte development, and metabolism in *M. polymorpha*.
Introduction

Serine, an amino acid highly accumulated in plants\textsuperscript{1}, is involved in cell signaling in response to various environmental stresses and the biosynthesis of various biomolecules, such as nitrogenous bases, phospholipids, and sphingolipids\textsuperscript{2–4}. In plants, the major serine synthesis pathway is the photorespiratory glycolate pathway in tissues exhibiting photorespiration\textsuperscript{5–7} (Supplementary Fig. S1). In this pathway, glycine decarboxylase complex and serine hydroxymethyltransferase (SHMT) convert glycine to serine\textsuperscript{8,9}. However, in dark environments and non-photosynthetic tissues, two other pathways are involved in serine synthesis, namely, the glycerate and phosphorylated pathways\textsuperscript{9,10}. The glycerate pathway starts with the dephosphorylation of 3-phosphoglycerate (3-PGA) by 3-PGA phosphatase in the cytosol or peroxisomes, followed by a sequence of reactions catalyzed by glycerate dehydrogenase (GDH) and alanine: hydroxypyruvate (serine: pyruvate) aminotransferase\textsuperscript{5,11–13}. On the other hand, the phosphorylated pathway from 3-PGA occurs in plastids and is catalyzed by 3-PGA dehydrogenase (PGDH), 3-phosphoserine aminotransferase (PSAT), and 3-phosphoserine phosphatase (PSP)\textsuperscript{9,14,15}.

The phosphorylated pathway is conserved in animals, plants, and bacteria\textsuperscript{4,16}. This pathway in plants is the only serine source for specific cell types and is essential for the embryo, pollen, male gametophyte, and postembryonic root development in Arabidopsis thaliana\textsuperscript{3,17–19}. The phosphorylated pathway of serine biosynthesis (PPSB) has been suggested to play a fundamental role in plant responses to various environmental stresses, such as low temperature, high salinity, and pathogens\textsuperscript{3,4,17,20}. Although the reason why plants take three routes for serine biosynthesis is unclear, serine production through the phosphorylated pathway is crucial in plant metabolism and development\textsuperscript{3,21}. The genes encoding PGDH are important checkpoints in PPSB as they are under tight transcriptional control and responsible for several physiological events\textsuperscript{3}. In A. thaliana, three PGDH isoforms (AtPGDH1, AtPGDH2, and AtPGDH3) are expressed in different organs/tissues and have different physiological functions. AtPGDH1 is the essential gene; its silencing causes developmental arrest in roots, embryos, pollen, and male gametophytes, while mutations in AtPGDH2 or AtPGDH3 showed no evident morphological phenotypes\textsuperscript{17–19,22}. 
However, the triple mutant *Atpgdh1/2/3* performed better on primary root growth than the
double mutant *Atpgdh1/2* but not *Atpgdh1/3*, suggesting knockout of *AtPGDH2* and
*AtPGDH3* had the opposite effect in an *Atpgdh1* mutant background. *AtPGDH2* has a
partially redundant role with *AtPGDH1*, while *AtPGDH3* seems to have additional functions
unrelated to serine synthesis\(^{23}\). In addition to playing a critical role in linking plant growth
with nitrogen metabolism\(^{24}\), *AtPGDH1* has a specific function in serine supply for the
biosynthesis of the auxin precursor tryptophan regulated by transcription factors *AtMYB34*
and *AtMYB51\(^{3,17}\). The biochemical properties of *AtPGDH* enzymes are different among
isoenzymes in terms of allosteric regulation by amino acids\(^{25}\). Considering that all land plant
species examined, except *Marchantia polymorpha* L., possess different types of PGDH
isoenzymes, duplication and functional diversification of the PGDH genes was necessary for
the evolution of land plants to adequately control the serine supply\(^{26,27}\).

*Marchantia polymorpha* is a model bryophyte species and a dioecious plant with a
haploid genome. *M. polymorpha* is increasingly used as a model plant for physiological,
genetic, epigenetic, metabolic, and evolutionary studies. Recent studies have clarified
metabolic responses to wounding stress\(^{28}\), cellular expansion and integrity\(^{29}\), reproductive
development\(^{30}\), as well as the mechanisms of spermatogenesis and embryogenesis\(^{31–35}\). Our
previous study characterized the sole PGDH enzyme, MpPGDH, a 65.6 KDa protein sharing
75–80% identity with *AtPGDH*\(^{27}\). MpPGDH has similar biochemical characteristics to
*AtPGDH1*, such as cooperative inhibition by *L*-serine and activation by *L*-alanine, *L*-valine,
*L*-methionine, *L*-homoserine, and *L*-homocysteine\(^{27}\).

In this study, we aim to clarify the function of *MpPGDH* and explore the specific
functions of serine from the phosphorylated pathway in a non-vascular plant *M. polymorpha*.
Our results revealed the particular importance of serine from the phosphorylated pathway on
vegetative growth, male gametogenesis, and sporophyte development in *M. polymorpha* and
that PPSB is involved in the metabolism of the tricarboxylic acid (TCA) cycle, amino acids,
nucleotides, and lipids, in a different manner under different light and CO\(_2\) conditions in *M.
polymorpha*. 
Results

Serine supply from the phosphorylated pathway in the dark is crucial for thallus growth

The expression pattern of MpPGDH was clarified using the proMpPGDH:GUS transgenic lines. In vegetative phase, MpPGDH was expressed in almost the entire gemma (Supplementary Fig. S2a) and displayed a linear expression pattern in the midribs from the center of the thallus to the apical notches in 1- to 4-week-old thalli (Supplementary Fig. 2b-d).

The knockout mutants of MpPGDH were generated by introducing the CRISPR/Cas9 constructs into sporelings (Supplementary Fig. S3a). The reduced transcript level of MpPGDH and no detectable level of MpPGDH protein were confirmed in both male and female Mppgdh mutants (Supplementary Fig. S3b, c).

Considering that photosynthesis may affect serine biosynthesis in plants, thalli were grown under 16-h light/8-h dark (L/D) and continuous light (CL) conditions for comparison. Under the L/D condition, male Mppgdh-1 and Mppgdh-2 lines were significantly small in thallus size and fresh weight (Fig. 1) compared with the wild type. However, the severe growth phenotype of Mppgdh mutants was resolved by the exogenous supplementation with serine in the medium (Fig. 1). In contrast, no difference was observed between the mutants and wild type under the CL condition, while M. polymorpha grew better under this condition than the L/D condition (Fig. 1). Similar growth phenotypes were observed in the female Mppgdh knockout mutants (Mppgdh-3, Mppgdh-4, and Mppgdh-5; Supplementary Fig. S4). For further experiments, Mppgdh-1 and Mppgdh-3 were used since the supply of serine restored the growth of these mutants to the wild-type levels.

Poor growth phenotype observed only under L/D conditions suggested the importance of the PPSB in the dark. Then, the transcript levels of some key genes involved in three serine biosynthesis pathways were analyzed in the thallus transferred from CL to dark conditions. In Supplementary Fig. S5, the expression ofMpPGDH and MpPSAT in the PPSB was significantly induced at 8 and 16 h post transfer to darkness, respectively. TheMpGDH and MpSHMT expression levels in the glycerate and glycolate pathways, respectively, were gradually reduced in darkness.
After 24 h in darkness, the tested genes, except for MpPSP whose expression was relatively stable under light and dark conditions, were repressed to low expression levels. These results suggest that the PPSB was enhanced and functioned as the primary serine synthesis pathway under dark conditions.

To determine whether poor growth of the mutant thalli under L/D conditions was attributed to insufficient endogenous serine supply, free amino acid contents in the thallus were measured. Compared with that in the wild type, no significant change was observed in the serine content in Mppgdh-1, while that in Mppgdh-3 was significantly decreased to 75% (Supplementary Fig. S6).

Overall, these results indicate that the perturbation of the PPSB impaired thallus growth under the L/D condition, revealing the crucial function of PPSB in the dark for thallus growth.

**MpPGDH-mediated serine synthesis is essential to sperm formation**

The growth phenotype of Mppgdh mutants was observed in the whole life cycle under the L/D condition to understand the importance of PPSB. The formation of gemma cups and gemmae in the Mppgdh mutants was normal, likewise those of wild types (Supplementary Fig. S7).

The male Mppgdh mutants developed umbrellalike antheridiophores (male reproductive branch) under sexual reproduction-inducing far-red irradiation conditions (Fig. 2a, b). Antheridiophore development is generally divided into five stages (Supplementary Fig. S8a). When water was dropped on the surface of the antheridiophores at stage 4, white sperm clusters originated from the antheridal pores in wild type (Fig. 2c). Notably, no white sperm cluster was observed in the Mppgdh mutants after water treatment (Fig. 2c and Supplementary Fig. S8b). When sperm cells in water were visualized via fluorescence staining of DNA, very few sperm cells were observed in the Mppgdh-1, while wild-type Tak-1 produced over 20 times more sperm cells than Mppgdh-1 (Fig. 2d).

To ascertain which process of sperm formation was impaired in the Mppgdh mutants, the sections of resin-embedded antheridia were analyzed using field emission scanning electron microscopy (FE-SEM) at the early-, middle-, and mature stages.
In wild-type Tak-1, spermatid mother cells were rectangular (Fig. 2e, left) and underwent diagonal cell division to produce spermatids (Fig. 2e, middle). In the spermatid stage, flagellar production was visible. Lastly, the spermatids differentiated into sperm cells (Fig. 2e, right). However, in the Mp\textit{pgdh} mutants, spermatids had different sizes and irregular shapes, and no subsequent diagonal cell division and sperm differentiation occurred (Fig. 2e).

The GUS staining of the male \textit{proMpPGDH:GUS} lines indicated that Mp\textit{PGDH} was expressed in the middle area of antheridial receptacles (Supplementary Fig. S2e). Regardless of the strong phenotype in spermatogenesis in the Mp\textit{pgdh} mutants, Mp\textit{PGDH} expression was not detectable in the antheridium that produced sperm cells (Supplementary Fig. S2g, h).

To clarify whether exogenous serine supplementation and continuous light rescue the defective spermatogenesis phenotype, the mutants grown under L/D + serine condition and CL condition were microscopically observed. Under L/D + serine conditions, the phenotype was partially rescued and the number of sperm cells in Mp\textit{pgdh-1} amounted to approximately 20% of that of the wild type (Supplementary Fig. S8c, d). Under CL conditions (Supplementary Fig. S8e, f), the wild type produced more sperm cells than L/D conditions (Fig. 2d). The mutant produced more sperm cells compared with under L/D condition, but the number was approximately 20% of that of the wild type under the CL condition (Supplementary Fig. S8f).

The FE-SEM images indicated that the cell division of spermatid mother cells in Mp\textit{pgdh-1} was rescued to some extent by serine supplement and CL, since diagonal cell divisions were visible in some regions in one antheridium (Supplementary Fig. S8g, h). The borders of the areas comprising cells in different states were clearly observed (Supplementary Fig. S8g, h), indicating that sperm differentiation was asynchronous in one antheridium in Mp\textit{pgdh-1}, while it is highly synchronous in the wild type\textsuperscript{37}. Partial recovery by exogenous serine supplement and CL might result in fewer number of sperm cells in the mutants compared with the wild type.

In the female Mp\textit{pgdh} mutants, no apparent morphological differences were observed in the formation of archegoniophores (female reproductive branch, Fig. 3a, b) and archegonia (Fig. 3c) under the L/D condition with far-red light, compared with that in the wild type.
GUS staining of the female *proMpPGDH:GUS* lines also indicated that *MpPGDH* was barely expressed in archegonial receptacles (Supplementary Fig. S2f). Overall, these results suggest that male gametogenesis required serine supply from the phosphorylated pathway.

**Knockout of *MpPGDH* in maternal genome affects sporophyte development**

Next, we observed the process on the archegoniophore of wild-type Tak-2 and *Mppgdh-3* after fertilization with sperm from wild-type Tak-1. In the Tak-2 x Tak-1 cross, yellow spores were generated and released from sporangia (Fig. 4a), whereas in the *Mppgdh-3* x Tak-1 cross, only empty involucres were developed (Fig. 4b). Cross-sectional images of the archesperical tissue show the detailed process of spore formation post fertilization (Fig. 4c-h). At 1-week post fertilization, a young sporophyte circularly surrounded by a calyptra and pseudoperianth was observed in both Tak-2 x Tak-1 (Fig. 4c) and *Mppgdh-3* x Tak-1(Fig. 4d), indicating that fertilization successfully occurred in *Mppgdh-3* x Tak-1 as well. In Tak-2 x Tak-1, sporophytes detached into the inner space of the capsule, and sporogenous cells began to differentiate at 2-week post fertilization (Fig. 4e). At 3-week post fertilization, sporocytes underwent meiosis to produce spores (Fig. 4g). In contrast, no sporophyte differentiation was observed in the front end of the seta in mutant (Fig. 3f, h), resulting in no spore production. Considering that paternal chromosomes are repressed during sporophyte formation (diploid phase), this phenotype was attributed to the lack of the functional *MpPGDH* in maternal genome. Interestingly, according to the Expression Database for *M. polymorpha*, the strongest expression of *MpPGDH* (Mapoly0030s0029) was observed in sporophyte. Under the CL conditions, the *Mppgdh-3* x Tak-1 zygotes produced spore-bearing structures, and these spores germinated and grew into healthy individuals (Supplementary Fig. S9).

These results indicate that the knockout of *MpPGDH* gene in maternal genome affected sporophyte development after fertilization.
DNA-dependent processes are impaired during gametogenesis in male Mp\textit{pgdh} mutants

To further understand how \textit{MpPGDH} knockout affects male gametogenesis at the molecular level, some sperm formation-related genes were selected based on reported RNA sequencing data\textsuperscript{36} to assess their transcript levels in antheridal receptacles using qRT-PCR analysis (Fig. 5).

We focused on DUO POLLEN 1 (DUO1), DUO1-ACTIVATED ZINC FINGER1 (DAZ1), and RWP-RK domain (RKD) containing transcription factors. DUO1 is a key MYB transcription factor that controls the emergence and maintenance of sperm differentiation in land plant lineages\textsuperscript{31,39,40}, whereas DAZ1 is a transcription factor acting downstream of DUO1. Many genes involved in male gamete differentiation are regulated by the DUO1-DAZ regulatory network\textsuperscript{32,41}. A single RKD containing transcription factor is required for egg and sperm formation in land plants\textsuperscript{37,42–44}. In our study, the expression of the three transcription factors was not influenced by \textit{MpPGDH} gene knockout (Fig. 5).

PROTAMINE-LIKE (Mp\textit{PRM}), ALPHA-TUBULIN 5 (Mp\textit{TUA5}), DYNEIN LIGHT CHAIN 7 (Mp\textit{LC7}), and CENTRIN 1 (Mp\textit{CEN1}) are functional proteins in sperm cells. \textit{MpPRM} encodes a protamine-like arginine-rich protein involved in chromatin compaction and nuclear morphogenesis\textsuperscript{36}. \textit{MpTUA5} encodes an antheridium-specific isoform of $\alpha$-tubulin\textsuperscript{36}. \textit{MpLC7} is a homolog of the \textit{LC7/Roadblock} gene family, first found in outer arm dynein of \textit{Chlamydomonas}\textsuperscript{45}. \textit{MpCEN1} encodes centrin (caltractin), which is required for centriole duplication and motility apparatus formation\textsuperscript{46,47}. The expression of these proteins is regulated by \textit{MpDUO1}\textsuperscript{31}. In \textit{Mppgdh-1}, regardless of unchanged \textit{MpDUO1} expression, the transcript level of \textit{MpPRM} was strongly reduced and that of \textit{MpCEN1} was slightly upregulated compared with that in Tak-1 (Fig. 5); however, \textit{MpTUA5} and \textit{MpLC7} transcript levels were unaffected.

High Mobility Group box (HMGBOX) proteins are architectural DNA-binding proteins in the nucleus and mitochondria, which regulate DNA-dependent processes, such as transcription, replication, and DNA repair\textsuperscript{48,49}. Among the five selected members of the
MpHMGBOX family, only MpHMGBOX4 exhibited significantly weaker expression in the MpPgdh mutants.

DNA topoisomerases (TOPs) are enzymes responsible for DNA torsional stress generated during replication and transcription. Among them, Top1α is involved in cell division and chromatin condensation in the process of synchronous spermatogenesis in Physcomitrium patens. Marchantia polymorpha possesses four members in the TOP family. In the MpPgdh mutants, MpTOP2 and MpTOP3α transcript levels were increased moderately.

Autophagy-mediated organelle remodeling in spermatogenesis is essential for male fertility. The known key functional autophagy-related genes, MpATG5, MpATG7, MpATG13, showed similar expression levels in Tak-1 and the MpPgdh mutants.

qRT-PCR results suggest that in the male MpPgdh mutants, DNA-dependent processes were impaired during early gametogenesis, resulting in significant disruption in sperm cell production, whereas autophagy for remodeling sperm cell structure during late gametogenesis was not responsible for the defect in spermatogenesis in the MpPgdh mutants.

MpPGDH knockout affects amino acid and nucleotide metabolism and the TCA cycle

To determine how MpPGDH knockout affects metabolism, we performed a widely targeted metabolome analysis using gas chromatography–triple quadrupole mass spectrometry (GC-QqQ-MS).

The MpPgdh mutants were grown under the L/D, CL, and L/D + serine conditions, and the metabolome in thalli was analyzed (Supplementary Fig. S10a). The principal component analysis (PCA) showed that the light condition did not significantly affect the metabolism of wide-type Tak-1 (Fig. 6a). In the PCA score plot, MpPgdh-1 formed the clusters separated from those of Tak-1 under L/D and CL conditions. The metabolomes of Tak-1 and MpPgdh-1 supplemented with serine were clearly separated from the others, indicating that abundant serine supplement in the medium greatly affected the metabolism in the wild type and mutant in a different manner (Fig. 6a). Similarly, serine supplementation greatly affected the metabolome of the female lines, while the wild-type Tak-2 and MpPgdh-3 were not separated in the PCA score plot under L/D and CL conditions without serine supplementation.
(Supplementary Fig. S11a). Thus, we mainly focused on the differentially accumulated metabolites (DAMs) (Supplementary Table S3) under L/D and CL conditions in the following analyses.

The Venn diagrams (Fig. 6b, c) and volcano plots (Supplementary Fig. S10b, c) show that 12 DAMs (e.g., malic acid and asparagine) were decreased in Mppgdh-1 commonly under L/D and CL conditions (Fig. 6b), while 7 DAMs (e.g., spermine and cytidine) were commonly increased under the two conditions (Fig. 6c). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that these DAMs were significantly enriched ($p < 0.01$) in “alanine, aspartate, and glutamate metabolism,” “TCA cycle,” and “beta-alanine metabolism” (Fig. 6d).

Similarly, DAMs in the female mutant Mppgdh-3 were also analyzed (Supplementary Fig. S11b, c). Under the CL condition, metabolic profiles of Mppgdh-3 were restored nearly to wild-type level, although the serine content was still significantly reduced in Mppgdh-3 (Supplementary Fig. S11c). Focusing on common DAMs in Mppgdh-1 and Mppgdh-3 under L/D conditions, 9 metabolites (e.g., malic acid, glutaric acid, and 2-amino adipic acid) decreased (Fig. 6e), and 4 metabolites (e.g., tyramine and guanine) (Fig. 6f) increased. These DAMs were enriched in “tyrosine metabolism” and “TCA cycle” (Fig. 6g).

We also analyzed metabolome of antheridial receptacles grown under L/D conditions, as Mppgdh-1 showed severe defect in spermatogenesis under this condition. The Mppgdh-1 and Tak-1 were separated in the PCA score plot (Fig. 7a), although metabolic profiles fluctuated slightly among replicate samples possibly due to the difficulty in clearly distinguishing antheridiophores at stages 3 or 4 when sampled (Supplementary Fig. S12a). The volcano plot in Fig. 7b indicates 6 increased metabolites and 4 decreased metabolites, of which lactitol, trehalose, maltose, butyl alcohol, cytidine, and dihydrouracil were common DAMs in thalli and antheridial receptacles.

These results indicate that MpPGDH knockout caused a significant metabolic change in the thallus and antheridiophore. Lack of functional MpPGDH mainly affected the TCA cycle and amino acid metabolism. Metabolism was affected even under the CL condition, where the Mppgdh mutants did not show a visible vegetative phenotype.
**MpPGDH knockout alters lipid profiles**

Since serine is a precursor of various lipid species, we analyzed the lipidome in the Mppgdh mutants using liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-QTOF-MS).

The lipidome in thalli of the Mppgdh mutants grown under the L/D, CL, and L/D + serine conditions were analyzed. In the PCA score plot (Fig. 8a, b), wild types and Mppgdh mutants were clearly separated under L/D and CL conditions, indicating a large difference in lipid profiles. Like the metabolic profiles, exogenous serine supplements greatly influenced the lipidome (Fig. 8a, b). Focusing on the differentially accumulated lipid classes (DALCs) (Supplementary Fig. S13), the Venn diagrams showed that lysodiacylglyceryl trimethylhomoserine (LDGTS) and stigmasterol esters (STSE) decreased under CL conditions (Fig. 8c), while sitosterol esters (SISE) increased under L/D conditions (Fig. 8d), commonly in male and female thalli.

Lipidome of antheridial receptacles grown under L/D conditions was also analyzed (Supplementary Fig. S12b). In the PCA score plot (Fig. 9a), Mppgdh-I and Tak-1 were separated from each other. Triacylglycerols (TG), oxidized triglycerides (OxTG), and phosphatidylethanolamines (PE) were present in high amounts, whereas oxidized phosphatidylcholines (OxPC) was present in low amounts in Mppgdh-I compared with that in Tak-1 (Fig. 9b).

Overall, these results suggest that MpPGDH knockout greatly influenced lipid composition in vegetative and reproductive growth phases of *M. polymorpha*.

**PPSB is the primary serine synthesis pathway when photorespiration is inhibited**

To estimate the importance of the glycolate pathway on growth and metabolism, the wild types and mutants were grown under L/D conditions in high CO₂ (3,000 ppm), which inhibited the glycolate pathway by suppressing photorespiration. GUS staining of proMpPGDH::GUS line showed a strong GUS signal in almost the entire thalli.
(Supplementary Fig. S2i), indicating that the MpPGDH expression was induced under high CO$_2$ conditions.

We analyzed thalli growth of the Mppgdh mutants. High CO$_2$ significantly increased the fresh weight of 14-day-old thalli in both wild types and the mutants, probably due to enhanced photosynthesis (Fig. 10a, b for males; Supplementary Fig. S14a, b for females). Growth of the mutants was greatly suppressed under high CO$_2$ conditions similar to under ambient CO$_2$ conditions. When serine was exogenously supplemented, suppressed growth of the mutants was recovered to some extent. However, the fresh weights of the mutants remained significantly lower than those of the wild type (Fig. 10b and Supplementary Fig. S14b). The serine content in Mppgdh-1 under high CO$_2$ + serine conditions was approximately 70 nmol/mg DW, which was 3.5-fold higher than that in Tak-1 under high CO$_2$ conditions without serine supplementation (Supplementary Fig. S15). Nevertheless, the Mppgdh-1 mutant under high CO$_2$ + serine conditions (approximately 100 mg FW, Fig. 10b) did not grow bigger than the wild type without serine supplementation (approximately 130 mg FW, Fig. 10b). This indicates that thallus growth did not depend only on total serine amount.

Reproductive growth was further observed under high CO$_2$ conditions. Wild types grew into maturity with healthy sexual reproduction branches in about two months with far-red light, while both male and female mutants were strongly impaired in growth and did not develop reproductive branches (Supplementary Fig. S16a). Interestingly, exogenous serine supplementation restored reproductive branch formation in Mppgdh mutants (Supplementary Fig. S16a), while spermatogenesis was not fully restored (Supplementary Fig. S16b). Considering that Mppgdh mutants developed reproductive branches under L/D and ambient CO$_2$ conditions but failed in sperm formation (Fig. 2a and 3a), the result indicated that enough serine supply from either the glycolate pathway or the PPSB is sufficient for reproductive branch development, while the PPSB is necessary to normal spermatogenesis in M. polymorpha.

Metabolism in vegetative growth under high CO$_2$ conditions was also investigated using metabolome and lipidome analyses. The PCA score plots (Fig. 10c, d) indicate that the
metabolic and lipid profiles of Mppgdh-1 were apparently different from those of Tak-1 under high CO₂ conditions, and the difference between Mppgdh-1 and Tak-1 became smaller when serine was exogenously supplemented. The volcano plots (Fig. 10e-h) show that high CO₂ conditions affected the accumulation of several metabolites and lipid classes in Mppgdh-1, and such perturbed metabolic status was restored by exogenous serine supplement. A similar trend was observed for Mppgdh-3 and Tak-2 (Supplementary Fig. S14c-h).

The metabolites and lipid classes whose accumulation were significantly affected by high CO₂ and MpPGDH knockout were identified via Venn diagrams. A total of 15 DAMs decreased (Supplementary Fig. S17a), while 3 increased (Supplementary Fig. S17b) commonly in male Mppgdh-1 and female Mppgdh-3. The KEGG pathway analysis indicates that these DAMs were enriched (p < 0.01) in the metabolite sets “TCA cycle,” “butanoate metabolism,” “alanine, aspartate and glutamate metabolism” (Supplementary Fig. S17c). In the case of lipidome, 15 DALCs decreased in male and female mutants under high CO₂ conditions (Supplementary Fig. S17d), while no lipid class was commonly increased. Decreased DALCs included serine-derived lipid classes, such as ceramide alpha-hydroxy fatty acid-phytospingosine (Cer_AP), hexosylceramide hydroxyfatty acid-sphingosine (HexCer_HS).

**Discussion**

**Serine homeostasis is essential for normal growth and development of M.polymorpha**

In plants, the photorespiratory glycolate pathway is the primary producer of serine during the day. MPGDH expression was induced when thallus was transferred to darkness (Supplementary Fig. S5) and grown under high CO₂ conditions (Supplementary Fig. S2i), suggesting a mechanism to activate the PPSB in situations where serine synthesis by the glycolate pathway is suppressed. Consistent with this, the suppressed growth of Mppgdh mutants (Fig. 1 and Supplementary Fig. S4) was specifically observed under L/D conditions. The growth phenotype was not correlated to the serine content in thalli (Supplementary Fig. S6). This result was consistent with the report that the A. thaliana double and triple mutants
of AtPGDHs showed drastic inhibition on aerial part growth, while the serine levels were not lower than those in the wild type\textsuperscript{23}.

Although Mppgdh-I absorbed a considerable amount of exogenously supplemented serine under high CO\textsubscript{2} conditions (Supplementary Fig. S15), it did not completely rescue the suppressed growth caused by inhibition of the phosphorylated and glycolate pathways (Fig. 10a, b and Supplementary Fig. S14a, b). This indicates that the growth defect induced by the lack of serine production via the PPSB and the glycolate pathway cannot be completely rescued by exogenous serine supplementation and that normal vegetative growth is supported by \textit{in vivo} homeostasis of serine synthesis, not only by the total amount of serine. Under CL conditions, serine was continuously synthesized from the glycolate pathway, which was sufficient for growth of the Mppgdh mutants (Fig. 1). Conversely, under L/D conditions, the lack of serine production by PPSB at night greatly suppressed thalli growth of the Mppgdh mutants (Fig. 1 and Supplementary Fig. S4), although serine in the mutants was mainly produced by the glycolate pathway during the day and amounted to almost the same level with wild types (Supplementary Fig. S6). These results further suggest that stable serine production throughout the day is necessary for vegetative growth.

Regarding gametogenesis, Mppgdh-I barely produced sperm cells under L/D conditions (Fig. 2c, d) but produced a certain number of sperm cells when supplemented with serine or grown under CL conditions (Supplementary Fig. S8c-f). MpPGDH was not expressed in the antheridium, where sperm cells are produced (Supplementary Fig. 2g, h). Zimmermann et al.\textsuperscript{24} proposed that externally supplied amino acids are metabolized similarly to those produced in plants. In our study, however, externally supplemented serine recuperated stagnant thallus growth but did not impaired spermatogenesis in Mppgdh-I. One possibility is that serine supplied by the PPSB is essential for sperm formation. Another possible reason is that exogenously supplemented serine is not fully transported to the organ/tissue where serine is required. The long-distance transport of serine to the antheridial receptacles or serine translocation from the synthesis site toward the demanding antheridium might be insufficient to support normal developmental processes. It remains unclear whether gametophyte of \textit{Marchantia} have photosynthate-conducting cells\textsuperscript{52}. Detailed spatiotemporal analysis of
amino acid contents would provide insights into the importance of serine homeostasis in development.

Under L/D + high CO$_2$ conditions, wild types developed reproductive branches, while the growth of Mp$pgdh$ mutants was arrested at a particular phase (Supplementary Fig. S16a). These findings suggest that serine supply only from the glycerate pathway, the possible third pathway of serine synthesis, is insufficient to maintain the sustained growth of $M$. polymorpha. To date, the existence of the glycerate pathway has been proven by genomic information; however, its functional significance remains elusive. Further studies on this pathway will provide more information on serine homeostasis in plants.

**PPSB disruption affects spermatogenesis and sporophyte development**

Sexual reproduction is an important developmental process for plants throughout their life cycle. In $A$. thaliana, At$PSP1$- and At$PGDH1$-knockout lines showed drastic impairment of pollen development and were sterile$^{17-19}$, indicating the importance of PPSB in pollen development. In a dioecious plant $M$. polymorpha, although the male Mp$pgdh$ mutants developed antheridiophores, sperm formation was significantly suppressed (Fig. 2a, c). MpDUO1 is the main transcription factor regulating the emergence and maintenance of sperm differentiation. In the Mp$DUO1$ knockout line, the diagonal division, which produces spermatids, occurred normally; however, subsequent sperm differentiation failed$^{31}$. In our study, knockout of Mp$PGDH$ had no significant influence on Mp$DUO1$ expression (Fig. 5). However, the expression of Mp$PRM$ and Mp$CEN1$, regulated by MpDUO1, was changed in Mp$pgdh$ mutants and the morphological phenotype of spermatogenous cells was more severe than that of Mp$DUO1$ knockout mutant. FE-SEM images showed that cell division from spermatid mother cells to spermatids was affected, and intranuclear structure was disrupted in Mp$pgdh$ mutants (Fig. 2e). This result suggests unknown regulatory mechanisms for spermatogenesis-related gene expression bypassing MpDUO1, which is affected by serine from the PPSB or in vivo serine homeostasis.

Among the known genes related to gametogenesis, the expression of Mp$HMBOX4$ and Mp$PRM$ (Mp$PROTAMINE-LIKE$) was almost completely repressed (Fig. 5). HMBOX
proteins typically contain a central DNA-binding domain that functions as a dynamic chromatin modulator during mitosis and meiosis. HMGBOXs weaken the binding of linker histone H1 to chromatin, thereby facilitating the access of transcription factors to their target sites. Protamines can replace histones during the last stages of sperm terminal differentiation in spermatogenesis to compact the sperm genome and prevent DNA damage. Histone modification to maintain stable nucleosomes or chromatin assembly may be impeded in the male Mppgdh mutants leading to failure in sperm formation, although the mechanism by which serine from PPSB influences gene expression and chromatin assembly remains unclear. RNA-seq and chromosome conformation capture technologies would help to identify other sperm formation-related genes affected by PPSB and capture the organizational structure of chromatin in male gametogenesis of Mppgdh mutants.

After fertilization with wild-type sperm of Tak-1, sporophyte development on the archegoniophore of the female mutant stopped at the seta stage only under the L/D condition (Fig. 4c-h). As egg cell was normally produced (Fig. 3c) and fertilization succeeded in the female Mppgdh mutant (Fig. 4c, d), internal serine supply seemed sufficient for these processes even without serine supply from PPSB. As the entire paternal genome is inactivated throughout embryogenesis in *M. polymorpha*, sporophyte from Mppgdh-3 x Tak-1 lacked functional PPSB. In liverworts, sporophyte is dependent on gametophyte for the supply of water and nutrients. It is possible that serine homeostasis in maternal gametophyte where sporophyte grows is essential for sporophyte development.

Taken together, the knockout of *MpPGDH* affected male gametogenesis and sporophyte development. Serine from PPSB affected male and female lines differently during sexual reproduction or development in *M. polymorpha*.

**MpPGDH knockout affects metabolism, including that of lipids**

Plants require a constant supply of serine for the biosynthesis of nucleic acids, amino acids, glutathione, and lipids. PPSB is involved in ammonium and sulfur assimilation in *A. thaliana*; therefore, it is reasonable to assume that a lack of serine from the PPSB would extensively affect metabolism in *M. polymorpha*. 
Our metabolome analysis identified the metabolites related to the tricarboxylic acid (TCA) cycle, such as aconitic acid, fumaric acid, and malic acid, as DAMs in Mp pgdh mutants (Fig. 6b, e). The result was consistent with the function reported in A. thaliana that PPSB represents a vital branching point in the metabolic flux between serine synthesis and the TCA cycle.

Among carbohydrates, the amounts of erythrulose and erythritol were greatly decreased in the Mp pgdh mutants (Fig. 10e and Supplementary Figs. S10b, c, S11b, and 14e). In yeast, these metabolites are derived from erythrose 4-phosphate in the pentose phosphate pathway. Although the biosynthesis pathway of these metabolites in plants is still unclear, our result suggests that the PPSB affects central carbon metabolism.

Asparagine was most reduced among the proteinogenic amino acids analyzed in the Mp pgdh mutants (Supplementary Fig. S6). The amounts of glutaconic acid and 2-aminoadipic acid, the intermediates of lysine degradation, were also reduced in the mutants (Fig. 6b, e and Supplementary Fig. S17a). The DAMs detected in this study were enriched in amino acid metabolism, especially alanine, aspartate and glutamate metabolism (Fig. 6d, g and Supplementary Fig. S17c), indicating that lack of the PPSB-mediated serine widely affects metabolisms of various amino acids.

Serine plays an indispensable role in the metabolism of one-carbon units. One of the main outputs of one-carbon metabolism is nucleotide biosynthesis. The amount of cytidine, a component of RNA, was increased, whereas that of dihydrouracil, an intermediate in the catabolism of uracil, was reduced in both thalli and antheridial receptacles of Mp pgdh-1 (Fig. 6b, c and Fig. 7b). These detected DAMs suggest that serine synthesis via the PPSB is related to nucleotide metabolism.

We clarified that the PPSB is strongly involved in lipid metabolism. Besides serving as structural components of membranes, lipids are involved as chemical messengers/signaling molecules in various developmental processes and responses to environmental stresses. Lipid species vary significantly among different plants. Improved analytical technologies and bioinformatics in lipidomics have enabled the investigation of highly complex composition...
of lipids in plants. In this study, we analyzed the lipid profile of *M. polymorpha* by using our cutting-edge lipidomics platform and discussed the link between serine metabolism and lipids.

Under L/D and high CO$_2$ conditions, lipid profiles were changed in Mppgdh mutants, and several DALCs were identified (Supplementary Figs. S13a, d, S14e, f, and Fig 10e, f). When serine was supplemented under these two conditions, the number of DALCs was greatly reduced (Supplementary Figs. S13b, e, S14g, h, and Fig 10g, h), indicating that lack of serine from the phosphorylated and the glycolate pathways caused significant changes in lipid profiles.

Two major serine-derived lipid classes are sphingolipids and phosphatidylethanolamines (PE). Sphingolipid biosynthesis starts from the condensation of serine with palmitoyl-CoA$^{66}$, while PE biosynthesis starts with the conversion of serine to ethanolamine$^{67}$. The contents of sphingolipids (phytosphingosine [PhytoSph], hexosylceramide hydroxyfatty acid-sphingosine [HexCer_HS], ceramide alpha-hydroxy fatty acid-phytosphingosine [Cer_AP], and ceramide non-hydroxyfatty acid-phytosphingosine [Cer_NP]) significantly decreased in Mppgdh mutants only under high CO$_2$ conditions (Fig. 10g and Supplementary Fig. S14f).

PE was also reduced in thalli of male mutant under ambient CO$_2$ but accumulated in antheridiophores (Fig. 8c and 9b).

Overall, our findings indicate that TCA cycle, amino acid metabolism, and nucleotide metabolism were among the primary pathways affected by serine deficiency from the PPSB. This study also revealed that lipid metabolism was affected by perturbation of serine metabolism.

**Proposed functions of PPSB**

The coexistence of different serine synthesis pathways complicates our understanding of the role of serine in plant development and metabolism. This study explained the specific function of PPSB in *M. polymorpha* and explored the relationship between three serine synthesis pathways in plants. Here, we present a model for functions of serine supplied by different pathways and serine homeostasis in *M. polymorpha* (Fig. 11). A large amount of serine from the photorespiratory glycolate pathway supports fundamental growth and
development, and PPSB is the main pathway for serine supply when the glycolate pathway
is inactive. Both pathways control serine homeostasis for normal growth and development.
PPSB is essential for sperm formation by affecting histone modification-related gene
expression, and the glycolate pathway cannot completely compensate for this function.
Serine from the glycolate pathway is sufficient for vegetative growth but not maintaining
metabolic homeostasis. Lack of PPSB-derived serine triggers metabolic and lipidomic
perturbation. Besides the TCA cycle, PPSB influences amino acid and nucleotide metabolism
and affects lipid profiles. Serine supply solely from the glycerate pathway is insufficient for
normal growth and development. Further research remains warranted to determine the
possible existence of the glycerate pathway and clarify its specific function and relationship
with the other two pathways.

In conclusion, serine from different biosynthesis pathways has different functions in
plants. This study demonstrated the significance of PPSB in vegetative growth and male
gametogenesis and the importance of serine homeostasis. Our findings provide novel insights
into the functional significance of serine from different pathways and why plants use three
pathways for serine synthesis. Further investigation remains warranted to elucidate the
mechanism underlying the influence of serine from PPSB on gene expression and
metabolic/lipidomic pathways.

**Online methods**

**Plant materials**

We used male strain Takaragaike-1 (Tak-1) and female strain Takaragaike-2 (Tak-2) as
wild types. To generate Mppgdh mutants using the CRISPR/Cas9 system, two gRNA
sequences gRNA1 (5'-GCGCATCGTGCAAGACCGCG-3', targeting in the 1st exon) and
gRNA2 (5'-TGAAGGCCGGTAAGTGCGCT-3', targeting at the junction of 1st intron and
1st exon) were designed to target MpPGDH. Next, the gRNA sequences were inserted into
pMpGE_EN03, and the gRNA expression cassette was transferred to pMpGE010 using LR
Clonase.
To generate the lines carrying GUS reporter gene driven by the promoter of MpPGDH (the proMpPGDH:GUS lines), the genomic sequence of the upstream region of MpPGDH (5000 bp) was amplified with PCR using the genomic DNA of Tak-1 as a template. Subsequently, the PCR product was ligated into pMpGWB104 to construct a pMpGWB104:proMpPGDH plasmid.

All plasmids were purified using LaboPass™ Plasmid Mini. The vectors (pMpGE010-MpPGDH-gRNA1, pMpGE010-MpPGDH-gRNA2, and pMpGWB104:proMpPGDH) were introduced into sporelings produced by crossing Tak-1 and Tak-2 via co-cultivation with an Agrobacterium strain harboring the vectors. After screening independent T₁ plants following an antibiotic selection step, we obtained 3–5 candidate lines for each plasmid in the male and female backgrounds selected via PCR using rbm27-F/rbm27-R and rhf73-F/rhf73-R, respectively. All primers used are listed in Supplementary Table S1.

**Growth conditions**

Wild-type and mutant *M. polymorpha* were cultivated on half-strength Gamborg’s B5 medium (pH = 5.5–5.6) containing 1% agar with or without 10 mM serine at 22 °C. We used three chambers, CL with air, 16-h light/8-h dark with air, and 16-h light/8-h dark with 3000 ppm CO₂, to grow plants. To induce sexual reproduction branches, we applied additional far-red light irradiation to each chamber. Nylon membranes were tiled onto the surface of the medium to ease the transfer of *M. polymorpha* from the agar medium.

Genetic crosses of *M. polymorpha* were performed under L/D and CL conditions as the video showed (https://www.youtube.com/watch?v=YFjfg-wsy0). After male and female lines developed sexual reproductive branches (approximately 1.5-month under L/D conditions, 1-month under CL conditions, and 2-month under L/D conditions in high CO₂), 50 μL of water was dropped on the surface of an antheridiophore (Stage 4) and left for 10 min. After pipetting five times, all water with sperm cells inside was taken and put to an immature archegoniophore. Post fertilization, 3–4 weeks were required to obtain mature sporangia that contain spores.
Western blot analysis of PGDH

Total protein from thalli of *M. polymorpha* was extracted in the sodium dodecyl sulfate (SDS) sample solution [2% (w/v) SDS, 62.5 mM Tris-HCl (pH = 6.8), 7.5% (v/v) glycerol, and 0.01% bromophenol blue]. After boiling for 5 min, the homogenate was centrifuged at 20,000 × g for 10 min, and the resulting supernatant was used as the protein sample. The protein concentration was determined using a BCA protein assay (Pierce). Equal amounts of proteins were separated using SDS polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene difluoride membrane. PGDH antibody was prepared using the recombinant protein of *Arabidopsis* PGDH1 as the antigen. Chemiluminescence was used for detection of horseradish peroxidase-conjugated secondary antibodies and visualized using LAS-3000 (Fujifilm).

Reverse transcription-quantitative PCR

Total RNA was extracted from the thalli and antheridial receptacles using ISOSPIN Plant RNA (NIPPON GENE). Next, total RNA (500 ng) was reverse-transcribed using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (TOYOBO). Quantitative real-time PCR was performed using THUNDERBIRD™ SYBR qPCR Mix (TOYOBO) in the StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR cycling conditions were 95 °C for 15 min, 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. The gene expression level was normalized to that of MpACT1 to obtain a relative expression level.

The biological samples were analyzed in triplicate. Primers used for qPCR are listed in Supplementary Table 2.

Hoechst staining of sperm cells

Stage 4 antheridiophores were used to assess sperm production. First, 50 μL water was dropped onto the surface of each antheridiophore and left for 10 min. After pipetting five times, 30 μL water was placed in a 1.5-mL tube with 1.5 μL bisBenzimid H33342 trihydrochloride (1 mg/mL) (Sigma-Aldrich). After staining for 10 min, 10 μL solution was
drawn to a C-Chip (NanoEnTek) to check fluorescence by confocal microscopy (OLYMPUS U-HGLGPS BX53).

**GUS histochemical assay**

The gemmae, thalli, antheridiophores, and archegoniophores of *proMpPGDH:GUS* lines were submerged in cold 90% acetone for at least 10 min. Next, the samples were washed twice with 50 mM sodium-phosphate buffer (pH = 7.0). Subsequently, the samples were transferred into 2 mL Eppendorf tubes or 25 mL centrifuge tube (VIOLAMP) with GUS staining buffer [50 mM Na-phosphate buffer (pH7.0), 0.1% Triton, 10mM EDTA, 0.5 mM K$_3$Fe(CN)$_6$, 0.5 mM K$_4$Fe(CN)$_6$, 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt)] and incubated at 37 °C under dark conditions (for approximately 16 h). Afterward, the stained samples were decolored and fixed in 70% ethanol overnight at 4 °C to remove chlorophyll and other plant pigments. Lastly, before observation under dissecting or light microscope (LEICA DFC450 C), the thalli were soaked in a solution [8 g choral hydrate: 3 mL water: 1 mL triglyceride] for 24 h to make them transparent.

**Electron microscopy of resin-embedded tissues and surface structure**

The samples were fixed with 4% paraformaldehyde and 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH = 7.4) for 2 h at 22 °C and overnight at 4 °C, then post-fixed with 1% osmium tetroxide in 50 mM cacodylate buffer for 3 h at room temperature. After dehydration in a graded methanol series (25, 50, 75, 90, and 100%), the samples were embedded in Epon812 resin (TAAB). Subsequently, ultrathin sections (100 nm) were cut using a diamond knife on an ultramicrotome (Leica EM UC7, Leica Microsystems, Germany) and placed on a glass slide. Afterward, the sections were stained with 0.4% uranyl acetate, followed by lead citrate solution, and coated with osmium using an osmium coater (HPC-1SW, Vacuum Device, Japan)$^{72}$. The coated sections were observed using FE-SEM (SU8220, Hitachi High-Tech, Japan) with a yttrium aluminum garnet backscattered electron detector at an accelerating voltage of 5 kV.
The uncoated samples were observed and imaged with a low-vacuum tabletop SEM (Hitachi TM3000, Japan) using backscattered electrons and an accelerating voltage of 15 kV.

Preparation and observation of histological sections

For the histological cross-sections, archegoniophore and antheridiophore were dissected and fixed overnight in formalin–acetic acid–alcohol (4% formalin, 5% acetic acid, 50% ethanol) at room temperature. Then, samples were dehydrated using a graded series of ethanol washes [50, 60, 70, 80, 90, and 95% (v/v); 60 min each] and stored overnight in 99.5% (v/v) ethanol at room temperature. Next, fixed specimens were embedded in Technovit resin (Kulzer), in accordance with the manufacturer's instructions, and sectioned using a microtome (RM2125 RTS; Leica Microsystems). Sections were stained with Toluidine Blue and photographed under a microscope (Leica DM6 B) connected to a CCD camera (DFC 7000T; Leica Microsystems).

Amino acid analysis by gas chromatography mass spectrometry

Free amino acids were extracted from 4 mg of a freeze-dried powder sample in 1 mL of extraction solvent (methanol: Milli-Q water = 4:1) placed in 2 mL sampling tubes containing 3 mm zirconia beads using a tube rotator for 10 min. After centrifugation at 14,000 rpm at 22 °C for 10 min, 900 μL of supernatant was transferred to another tube. Next, the amino acids were extracted from the residues three more times, and the four extracted solutions were mixed. The 25 μg $^{13}$C-labeled amino acids (Cambridge Isotope Laboratories) in 3.6 mL extracted solution were derivatized using the EZ: faast™ for free amino acid analysis using GC-MS (Phenomenex). Afterward, 1 μL of the solution was injected using an AOC-20i auto-injector and subjected to amino acid analysis using GCMS-QP2010 Plus (Shimadzu Corporation). The absolute concentrations of the amino acids were calculated based on those of $^{13}$C-labeled amino acids.

Widely targeted metabolome analysis using GC-QqQ-MS
For widely targeted metabolome analysis, a 4 mg freeze-dried powder sample was mixed with 1 mL extraction solvent (80% methanol and 0.1% formic acid) in 2 mL sampling tubes containing 3 mm zirconia beads on Shake Master Neo (BMS, Tokyo, Japan). After centrifugation at 100,000 rpm at 22 °C for 1 min, 200 μL of supernatant was transferred to 1.5 mL tubes containing 200 μL extraction solvent and 20 μL adonitol (0.2 mg/mL). Three blank controls (extraction solvent) and 20 quality control (QC) samples (mixed test samples) were also prepared. After vortexing, the solvents in the tubes were evaporated using a rotary evaporator (Thermo Scientific, Savant, SPD121P, & UVS800DDA) for 3 h, following which 100 μL MOX reagent (2% methoxyamine·HCl in pyridine) was added to tubes to dissolve the samples at 30 °C and 1,200 rpm for 6 h. Subsequently, 50 μL MSTFA 1% TMCS (Thermo) was added to the tubes and incubated at 37 °C and 1200 rpm for 30 min. After centrifugation at 5,000 rpm for 1 min, 50 μL supernatant was collected and analyzed using GC-QqQ-MS (AOC-5000 Plus with GCMS-TQ8040, Shimadzu Corporation). All samples were tested in random order. Two QC samples were injected at regular intervals (every six test samples) throughout the analytical run for continuous recalibration. Raw data were collected using GCMS solution software (Shimadzu Corporation). Lastly, quality-filtered metabolites were selected with signal-to-noise ratio > 3 and QC relative standard deviation < 30%. Calculation and normalization of peak area values were conducted using MRMPROBS and LOWESS/Spline normalization tools as described previously. Detailed GC-MS/MS parameters and MRM transitions for widely targeted analysis have been previously described.

Lipidome analysis using LC-QTOF-MS

Five mg of freeze-dried powder samples were placed in a 2 mL centrifuge tube, mixed with an 800 μL of extraction solution (methyl tert-butyl ether/methanol = 3/1 (v/v) containing 1 μM of 1,2-didecanoyl-sn-glycero-3-phosphocholine, Sigma-Aldrich), and extracted by shaking at 900 rpm at 4 °C for 5 min on Shake Master Neo (BMS, Tokyo, Japan) using zirconia beads. Subsequently, 250 μL of water was added to the homogenate. After vigorous stirring on a vortex mixer and dark incubation for 15 min on ice, the homogenate was
centrifuged at 1,000 × g for 5 min. Afterward, the 200 μL of upper layer was transferred to a new 1.5 mL microcentrifuge tube. Next, the organic phase was evaporated to dryness using a centrifugal concentrator (ThermoSavant SPD2010, Thermo Fisher Scientific) at room temperature. The residue was dissolved in 250 μL of ethanol and centrifuged at 10,000 × g for 15 min. The supernatant was subjected to LC-MS/MS analysis. The dataset was analyzed using MS-DIAL version 4.80. The data processing parameters of minimum amplitude (for peak picking) and retention time tolerance (for peak alignment) were set to 100 and 0.1 min, respectively; however, the default parameters were used for the others. The annotation results were manually curated by considering the basis of the equivalent carbon number model of lipids, in which the elution behavior of molecules in reverse phase LC depends on the length of acyl chains and the number of double bonds in lipids. The representative adduct form used for lipid quantification was previously described. Lastly, the peak height was used for lipid quantification, and the total amount of each lipid class (including lipids with different acyl chains) was calculated and used for further analysis.

**Data visualization**

Volcano plots and KEGG pathway analysis were performed at MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/home.xhtml). Venn diagrams were created by Webtools (https://bioinformatics.psb.ugent.be/webtools/Venn/). All figures were optimized by GraphPad Prism9.0 software (https://www.graphpad.com/).
References


56. Bao, J. & Bedford, M. T. Epigenetic regulation of the histone-to-protamine transition


70. Fujisawa, M. *et al.* Isolation of X and Y Chromosome-Specific DNA Markers From a Liverwort. &lt;em&gt;Marchantia polymorpha&lt;/em&gt;, by Representational


**Acknowledgments**

We greatly appreciate Drs. Mie Shimojima (Tokyo Institute of Technology) and Hiroyuki Imai (Konan University) for discussion on lipids, Mr. Kouji Takano (RIKEN Center for Sustainable Resource Science) for his help on preparing LC-MS/MS samples, Dr. Hideya Fukuzawa (Kyoto University) for his assistance with high CO\textsubscript{2} experiments, Ms. Yoriko Matsuda (Kyoto University) for her technical assistance with construction of CRISPR/Cas9 lines, and Ms. Junko Takanobu (RIKEN Center for Sustainable Resource Science) for her help in preparing culture medium and weighing samples. We are grateful to Graduate Program of Transformative Chem-Bio Research (GTR) program in Nagoya University to support this study.

**Funding**

This work was supported in part by JSPS KAKENHI grant numbers 25113010 and 20H04852 to M.Y.H.

**Authors’ contributions**

**Competing interests**

The authors declare that they have no conflicts of interest.

**Figure legends**

**Fig. 1: Thallus growth in the male Mppgdh mutants.**

(a) Plants grown on \(\frac{1}{2}\) B5 agar medium for 14 days with or without serine supplementation under 16 h light/8 h dark (L/D) or continuous light (CL) conditions. Scale bars = 1 cm. (b) The fresh weight of Mppgdh-1, Mppgdh-2, and wild-type Tak-1. Data represent means ± SD of six biological replicates (n = 6). One-way ANOVA followed by Tukey’s test (\(p < 0.05\)) was performed in each group; columns with the same letter are not significantly different.

**Fig. 2: Male gametogenesis in the Mppgdh mutants.**

(a) Images of antheridial receptacles of Tak-1, Mppgdh-1, and Mppgdh-2 grown under L/D conditions. Scale bar = 1 cm. (b) The fresh weight of antheridial receptacles in (a). Data represent means ± SD of five biological replicates (n = 5). One-way ANOVA followed by Tukey’s test (\(p < 0.05\)) was performed (n.s., no significant difference). (c) The appearance of sperm clusters. The white sperm clusters (red arrowhead) were visible 10 min after dropping 50 μL water on the surface of antheridial receptacles. Scale bars = 1 mm. (d) Fluorescent staining of the sperm cells. The sperm cells in 10 μL water taken from (c) were visualized...
via Hoechst staining. The numbers of sperm cells in 1 μL water were counted (n = 3). Asterisk indicates statistically significant difference (p < 0.01) using Student’s t-test. (e) Field emission scanning electron microscopy (FE-SEM) images showing the process of *M. polymorpha* male gamete development. Cells in an early-stage antheridium (left), a middle-stage antheridium (middle), and a mature antheridium (right) are shown. The white arrowhead indicates the flagella. C, cytoplasm; N, nucleus; F, flagella; M, mitochondria. Scale bars = 5 μm.

**Fig. 3: Oogenesis in the Mppgdh mutants.**
(a) Images of the archegonial receptacles of Tak-2 and Mppgdh-3 grown under L/D condition. Scale bars = 1 mm. (b) The fresh weight of archegonial receptacles of Tak-2 and Mppgdh-3. Data represent means ± SD of 11 biological replicates (n = 11). Student’s t-test was performed (n.s., no significant difference). (c) The cross-section images of archegonia. Scale bars = 1 μm. E, egg cell; N, neck.

**Fig. 4: Sporulation in the female Mppgdh mutant following fertilization with sperm from the wild type.**
(a), (b) The sporulation on Tak-2 (a) and Mppgdh-3 (b) approximately 1 month post fertilization with sperm from Tak-1. Images are representative of three archegoniophores. Scale bars = 1 mm. (c)-(h) The cross-section images of sporophytes at 1-week (c, d), 2-week (e, f), and 3-week (g, h) post fertilization. Tak-2 x Tak-1 (c, e, g), Mppgdh-3 x Tak-1 (d, f, h). Scale bars = 1 μm. C, calyptra; F, foot; S, seta; Sp, sporangium.

**Fig. 5: Expression of the spermatogenesis-related genes in antheridial receptacles.**
The transcript levels of the genes with known functions in spermatogenesis were determined in the antheridial receptacles of Tak-1, Mppgdh-1, and Mppgdh-2 grown under L/D conditions using real-time polymerase chain reaction (qRT-PCR) (n = 3). MpACT1 was used as an internal control. Fold change to the expression level in Tak-1 was calculated and presented as a heatmap. Different letters indicate significant differences between the lines.
Columns with the same letter are not significantly different (Tukey’s test following ANOVA, $p < 0.05$, n.s., no significant difference).

**Fig. 6: Changes in metabolome in 14-day-old thalli of the Mppgdh mutants.**
(a) PCA score plot of Tak-1 and Mppgdh-1 thallus samples grown under L/D, L/D + serine, and CL conditions ($n = 4$). (b), (c) Venn diagrams showing the number of significantly decreased metabolites (b) and increased metabolites (c) in thalli of Mppgdh-1. (d) KEGG pathway enrichment of common DAMs under L/D and CL conditions shown in (b) and (c). (e), (f) Venn diagrams showing the number of significantly decreased metabolites (e) and increased metabolites (f) in thalli of Mppgdh-1 and Mppgdh-3 under L/D conditions. (g) KEGG pathway enrichment analysis of common DAMs shown in (e) and (f). In (d) and (g), vertical and horizontal axes indicate the metabolite set and the value of $–\log_{10}(p$-value), respectively. The bubble size corresponds to the enrichment ratio. The color bar indicates the corrected $p$-value; yellow and navy blue represent higher and lower values, respectively.

**Fig. 7: Changes in metabolome in antheridial receptacles of Mppgdh-1.**
(a) PCA score plot of the antheridial receptacle (stage 4) samples grown under L/D conditions ($n = 5$). (b) Volcano plot showing the DAMs in antheridial receptacles of Mppgdh-1. Red dots and blue squares represent significantly increased ($p$-value $< 0.01$, fold change $> 2$) and decreased ($p$-value $< 0.01$, fold change $< 0.5$) metabolites, respectively, in Mppgdh-1. Black triangles represent no significant differences between Tak-1 and Mppgdh-1.

**Fig. 8: Changes in lipidome in 14-day-old thalli of the Mppgdh mutants.**
(a), (b) PCA score plots of male (a) and female (b) thallus samples grown under L/D, L/D + serine, and CL conditions ($n = 3$). (c), (d) Venn diagrams showing the significantly decreased (c) and increased (d) lipid classes in thalli of Mppgdh-1 and Mppgdh-3 under L/D and CL conditions.

**Fig. 9: Changes in lipidome in antheridial receptacles of Mppgdh-1.**
(a) PCA score plot of the antheridial receptacle samples grown under L/D condition (n = 4).

(b) Volcano plot showing the DALCs in antheridial receptacles of Mppgdh-1. Red dots and blue squares represent significantly increased (p-value < 0.05, fold change > 2) and decreased lipid classes (p-value < 0.05, fold change < 0.5) lipid classes, respectively, in Mppgdh-1. Black triangles represent no significant differences between Tak-1 and Mppgdh-1. Abbreviations are defined in Supplemental Table S4.

Fig. 10: Growth and metabolic phenotypes of male Mppgdh mutants under high CO2 conditions.

(a) Plants grown on ½ B5 agar medium for 14 days under L/D conditions in ambient CO2 (400 ppm) or high CO2 (3,000 ppm) with or without serine supplementation. Scale bars = 1 cm. (b) The fresh weight of Tak-1 and Mppgdh mutants. Data represent means ± SD of six biological replicates (n = 6). One-way ANOVA followed by Tukey’s test was performed (p < 0.05) in each growth condition; columns with the same letter indicate no significant differences. Student’s t-test was performed in each line grown under ambient CO2 and high CO2 conditions. Asterisks indicate statistically significant differences (Student’s t-test, *p < 0.05, **p < 0.01). (c), (d) PCA score plots of the metabolome (c) and lipidome data (d) in 14-day-old thalli of Tak-1 and Mppgdh-1 grown under high CO2 conditions with or without serine supplementation (n = 4). (e)-(h) Volcano plots showing DAMs (e, f) and DALCs (g, h) in Mppgdh-1 under the two growth conditions. Red dots and blue squares represent significantly increased (p-value < 0.01, fold change > 2) and decreased (p-value < 0.01, fold change < 0.5) metabolites or lipid classes, respectively, in Mppgdh-1. Black triangles represent no significant differences between Tak-1 and Mppgdh-1.

Fig. 11: Proposed model of serine homeostasis in M. polymorpha.

In M. polymorpha, three pathways are involved in serine synthesis. The phosphorylated pathway is the primary serine synthesis pathway when the glycolate pathway is inactive in dark or in high CO2 levels. Phosphorylated and glycolate pathways maintain stability in vivo serine homeostasis for normal growth and development. The phosphorylated pathway plays
a unique role in male gametogenesis by affecting the expression of chromatin assembly-
related genes (MpPRM and MpHMGBOX4) during spermatogenesis. Phosphorylated
pathway of serine biosynthesis (PPSB) disruption causes metabolic and lipidomic disorders.
The existence and function of the glycerate pathway remain unclear (dash line). The
mechanism via which serine affects gene expression is elusive (dotted line).

**Accession numbers**
Sequence data from this article can be found in the GenBank/EMBL data libraries under the
accession numbers: MpPGDH (Mp8g16970), MpACT1 (Mp6g10990), MpPSAT
(Mp1g15430), MpPSP (Mp2g10500), MpSHMT (Mp1g09830), MpGDH (Mp2g07580),
MpDUO1 (Mp1g13010), MpDAZI (Mp4g11380), MpMID (Mp1g11830), MpRKD
(Mp3g04030), MpPRM (Mp3g14390), MpTUA5 (Mp4g08430), MpLC7 (Mp6g01560),
MpCEN1 (Mp1g00710), MpHMGBOX1 (Mp8g07450), MpHMGBOX2 (Mp2g12330),
MpHMGBOX3 (Mp8g16760), MpHMGBOX4 (Mp2g04030), MpHMGBOX5 (Mp7g16870),
MpTOP1 (Mp6g05370), MpTOP2 (Mp3g13420), MpTOP3α (Mp1g02630), MpTOP3β
(Mp1g10070), MpATG5 (Mp1g12840), MpATG7 (Mp2g07850), MpATG13 (Mp7g03210).
Figure 1

Thallus growth in the male Mppgdh mutants. (a) Plants grown on ½ B5 agar medium for 14 days with or without serine supplementation under 16 h light/8 h dark (L/D) or continuous light (CL) conditions. Scale bars = 1 cm. (b) The fresh weight of Mppgdh-1, Mppgdh-2, and wild-type Tak-1. Data represent means ±
SD of six biological replicates (n = 6). One-way ANOVA followed by Tukey’s test (p < 0.05) was performed in each group; columns with the same letter are not significantly different.

Figure 2

Male gametogenesis in the Mppgdh mutants. (a) Images of antheridial receptacles of Tak-1, Mppgdh-1, and Mppgdh-2 grown under L/D conditions (Scale bar = 1 cm). (b) The fresh weight of antheridial receptacles in (a). Data represent means ± SD of five biological replicates (n = 5). One-way ANOVA followed by Tukey’s test (p < 0.05) was performed (n.s., no significant difference). (c) The appearance of sperm clusters. The white sperm clusters (red arrowhead) were visible 10 min after dropping 50 μL water
on the surface of antheridal receptacles. Scale bars = 1 mm. (d) Fluorescent staining of the sperm cells. The sperm cells in 10 μL water taken from (c) were visualized via Hoechst staining. The numbers of sperm cells in 1 μL water were counted (n = 3). Asterisk indicates statistically significant difference (p < 0.01) using Student’s t-test. (e) Field emission scanning electron microscopy (FE-SEM) images showing the process of M. polymorpha male gamete development. Cells in an early-stage antheridium (left), a middle-stage antheridium (middle), and a mature antheridium (right) are shown. The white arrowhead indicates the flagella. C, cytoplasm; N, nucleus; F, flagella; M, mitochondria. Scale bars = 5 μm.

Figure 3

Oogenesis in the Mppgdh mutants. (a) Images of the archegonial receptacles of Tak-2 and Mppgdh-3 grown under L/D conditions. Scale bars = 1 mm. (b) The fresh weight of archegonial receptacles of Tak-2 and Mppgdh-3. Data represent means ± SD of 11 biological replicates (n = 11). Student’s t-test was
Sporulation in the female Mppgdh mutant following fertilization with sperm from the wild type. (a), (b) The sporulation on Tak-2 (a) and Mppgdh-3 (b) approximately 1-month post fertilization with sperm from Tak-1. Images are representative of three archegoniophores. Scale bars = 1 mm. (c)-(h) The cross-section images of sporophytes at 1-week (c, d), 2-week (e, f), and 3-week (g, h) post fertilization. Tak-2 x Tak-1 (c, e, g), Mppgdh-3 x Tak-1 (d, f, h). Scale bars = 1 μm. C, calyptra; F, foot; S, seta; Sp, sporangium.

(c) The cross-section images of archegonia. Scale bars = 1 μm. E, egg cell; N, neck.
Figure 5

Expression of the spermatogenesis-related genes in antheridial receptacles. The transcript levels of the genes with known functions in spermatogenesis were determined in the antheridial receptacles of Tak-1, Mppgdh-1, and Mppgdh-2 grown under L/D conditions using real-time polymerase chain reaction (qRT-PCR) (n = 3). MpACT1 was used as an internal control. Fold change to the expression level in Tak-1 was calculated and presented as a heatmap. Different letters indicate significant differences between
Figure 6

Changes in metabolome in 14-day-old thalli of the Mppgdh mutants. (a) PCA score plot of Tak-1 and Mppgdh-1 thallus samples grown under L/D, L/D + serine, and CL conditions (n = 4). (b), (c) Venn diagrams showing the number of significantly decreased metabolites (b) and increased metabolites (c) in thalli of Mppgdh-1. (d) KEGG pathway enrichment of common DAMs under L/D and CL conditions shown.
in (b) and (c). (e), (f) Venn diagrams showing the number of significantly decreased metabolites (e) and increased metabolites (f) in thalli of Mppgdh-1 and Mppgdh-3 under L/D conditions. (g) KEGG pathway enrichment analysis of common DAMs shown in (e) and (f). In (d) and (g), vertical and horizontal axes indicate the metabolite set and the value of $-\log_{10}(p\text{-value})$, respectively. The bubble size corresponds to the enrichment ratio. The color bar indicates the corrected p-value; yellow and navy blue represent higher and lower values, respectively. Dopa, 3,4-dihydroxyphenylalanine; GABA, γ-aminobutyric acid.

Figure 7

Changes in metabolome in antheridial receptacles of Mppgdh-1. (a) PCA score plot of the antheridial receptacle (stage 4) samples grown under L/D conditions (n = 5). (b) Volcano plot showing the DAMs in antheridial receptacles of Mppgdh-1. Red dots and blue squares represent significantly increased (p-value < 0.01, fold change > 2) and decreased (p-value < 0.01, fold change < 0.5) metabolites, respectively, in Mppgdh-1. Black triangles represent no significant differences between Tak-1 and Mppgdh-1.
Figure 8

Changes in lipidome in 14-day-old thalli of the Mppgdh mutants. (a), (b) PCA score plots of male (a) and female (b) thallus samples grown under L/D, L/D + serine, and CL conditions (n = 3). (c), (d) Venn diagrams showing the significantly decreased (c) and increased (d) lipid classes in thalli of Mppgdh-1 and Mppgdh-3 under L/D and CL conditions.
Figure 9

Changes in lipidome in antheridial receptacles of Mppgdh-1. (a) PCA score plot of the antheridial receptacle samples grown under L/D condition (n = 4). (b) Volcano plot showing the DALCs in antheridial receptacles of Mppgdh-1. Red dots and blue squares represent significantly increased (p-value < 0.05, fold change > 2) and decreased (p-value < 0.05, fold change < 0.5) lipid classes, respectively, in Mppgdh-1. Black triangles represent no significant differences between Tak-1 and Mppgdh-1. Abbreviations are defined in Supplemental Table S4.
Figure 10

Growth and metabolic phenotypes of male Mppgdh mutants under high CO2 conditions. (a) Plants grown on ½ B5 agar medium for 14 days under L/D conditions in ambient CO2 (400 ppm) or high CO2 (3000 ppm) with or without serine supplementation. Scale bars = 1 cm. (b) The fresh weight of Tak-1 and Mppgdh mutants. Data represent means ± SD of six biological replicates (n = 6). One-way ANOVA followed by Tukey's test was performed (p < 0.05) in each growth condition; columns with the same letter indicate no significant differences. Student's t-test was performed in each line grown under ambient CO2 and high CO2 conditions. Asterisks indicate statistically significant differences (Student's t-test, *p < 0.05,
**p < 0.01). (c), (d) PCA score plots of the metabolome (c) and lipidome data (d) in 14-day-old thalli of Tak-1 and Mppgdh-1 grown under high CO2 conditions with or without serine supplementation (n = 4). (e)-(h) Volcano plots showing DAMs (e, f) and DALCs (g, h) in Mppgdh-1 under the two growth conditions. Red dots and blue squares represent significantly increased (p-value < 0.01, fold change > 2) and decreased (p-value < 0.01, fold change < 0.5) metabolites or lipid classes, respectively, in Mppgdh-1. Black triangles represent no significant differences between Tak-1 and Mppgdh-1.

Figure 11

Proposed model of serine homeostasis in M. polymorpha. In M. polymorpha, three pathways are involved in serine synthesis. The phosphorylated pathway is the primary serine synthesis pathway when the glycolate pathway is inactive in the dark or in high CO2 levels. Phosphorylated and glycolate pathways maintain stability in vivo serine homeostasis for normal growth and development. The phosphorylated pathway plays a unique role in male gametogenesis by affecting the expression of chromatin assembly-related genes (MpPRM and MpHMGBOX4) during spermatogenesis. Phosphorylated pathway of serine biosynthesis (PPSB) disruption causes metabolic and lipidomic disorders. The existence and function of the glycerate pathway remain unclear (dashed line). The mechanism via which serine affects gene expression is elusive (dotted line).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.pdf
- SupplementaryTableS3.xlsx