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Marchantia polymorpha MpPP2A-A Gene Cloning and Gene Knockout Mutant Construction Post-print

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Abstract

Protein phosphatase 2A (PP2A) is a serine-threonine protein phosphatase that participates in biological processes such as plant growth and development through dephosphorylating substrate proteins. Marchantia polymorpha is an emerging model plant with many advantages including a small genome and low gene redundancy. To investigate the regulatory mechanism of PP2A in plant growth, this study employed Marchantia polymorpha as the research object, cloned the full-length coding region of the MpPP2A-A subunit (MpPP2A-A), analyzed the tissue expression pattern of the MpPP2A-A gene using bioinformatics software and real-time fluorescent quantitative PCR technology, and constructed knockout mutants of the MpPP2A-A gene. The results showed: (1) The full-length coding region of the MpPP2A-A gene is 1,761 bp, encoding 586 amino acids, containing 3 domains, and lacking a signal peptide. (2) Amino acid sequence alignment results indicated that PP2A-A is relatively conserved during plant evolution. (3) Real-time fluorescent quantitative PCR revealed that the expression of the MpPP2A-A gene gradually decreased in the apical notch, thallus, and gemma cup. (4) Three independent mutant lines were successfully obtained via CRISPR/Cas9 technology; statistical analysis demonstrated that the gemma area of mutants was significantly reduced compared with wild-type Tak1, and their morphology was abnormal. These results indicate that the MpPP2A-A gene plays an important role in the growth process of Marchantia polymorpha gemmae, laying a foundation for further investigation of its molecular mechanism in regulating plant growth and development.

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Full Text

Cloning of the MpPP2A-A Gene and Construction of Knockout Mutants in Marchantia polymorpha

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Abstract: Protein phosphatase 2A (PP2A) is a serine/threonine protein phosphatase that participates in various biological processes such as plant growth and development through dephosphorylation of substrate proteins. *Marchantia polymorpha* is an emerging model plant with numerous advantages including a small genome and low gene redundancy. To investigate the regulatory mechanisms of PP2A in plant growth, this study cloned the full-length coding region of the MpPP2A-A subunit (MpPP2A-A) in *M. polymorpha*, analyzed its tissue-specific expression patterns using bioinformatics software and real-time quantitative PCR, and constructed knockout mutants of the MpPP2A-A gene. The results revealed: (1) The full-length coding region of MpPP2A-A is 1,761 bp, encoding 586 amino acids with three structural domains and no signal peptide. (2) Amino acid sequence alignment demonstrated that PP2A-A is relatively conserved during plant evolution. (3) Real-time quantitative PCR showed that MpPP2A-A expression decreases sequentially in the apical notch, thallus, and gemma cup. (4) Three independent mutant lines were successfully obtained via CRISPR/Cas9 technology, and statistical analysis revealed that gemma area in mutants was significantly reduced compared with wild-type Tak1, with abnormal morphology. These findings demonstrate that MpPP2A-A plays an important role in gemma growth in *M. polymorpha*, establishing a foundation for further investigation into the molecular mechanisms by which PP2A regulates plant growth and development.

Keywords: PP2A, *Marchantia polymorpha*, gene cloning, CRISPR/Cas9, vector construction

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Introduction

Protein phosphorylation and dephosphorylation modifications play crucial roles in cellular signal transduction. While researchers have long focused on protein kinases in phosphorylation, studies on dephosphorylation have been relatively limited. Protein phosphatase 2A (PP2A), a highly conserved and functionally diverse serine/threonine phosphatase, comprises numerous oligomeric enzymes (Virshup, 2000). Structurally, PP2A forms a heterotrimeric complex composed of a scaffold subunit (A), a regulatory subunit (B), and a catalytic subunit (C), which confers greater flexibility and functional diversity. The A and C subunits are ubiquitous and highly conserved, whereas B subunits exhibit more specific expression patterns. PP2A is essential for plant growth, development, and immune regulation (Lillo et al., 2014), participating in multiple physiological processes including ABA response (Punzo et al., 2018), auxin transport (Michniewicz et al., 2007), brassinosteroid signaling (Tang et al., 2011), ethylene signaling (Zhu et al., 2021), salt stress response (Zhao et al., 2019), and flowering (Heidari et al., 2013), particularly in hormone regulation and stress responses (Chen et al., 2014).

Current research on plant PP2A has primarily focused on *Arabidopsis thaliana*, which possesses three PP2A-A genes, seventeen PP2A-B genes, and five PP2A-C genes (Farkas et al., 2007). These genes participate in various hormone signaling pathways and cell differentiation processes. The PP2A-A subunit encoded by the RCN1 gene plays an important role in auxin transport, and RCN1 deficiency causes root curling, abnormal hypocotyl hook formation, uneven cell length, defects in gravitropic response, and polar auxin transport (Deruère et al., 1999). However, functional redundancy among the three PP2A-A subunit-encoding genes in *Arabidopsis* hinders deeper investigation, and the low transmission efficiency of double or triple mutants significantly prolongs experimental cycles, leaving the specific regulatory mechanisms unclear.

Marchantia polymorpha, as an early diverging land plant, offers advantages including easy propagation, short life cycle, small genome size (~280 Mb), and predominantly single-copy genes (Bowman et al., 2017). In recent years, transformation techniques (Kubota et al., 2013; Tsuboyama et al., 2018) and targeted genome modification (Sandler et al., 2023) have matured, establishing *M. polymorpha* as a model organism for studying plant evolution, molecular biology, cell biology, and development (Naramoto et al., 2022; Bowman et al., 2022; Kohchi et al., 2021; Ishizaki et al., 2015). Compared with *Arabidopsis*, *M. polymorpha* exhibits low gene redundancy, with only a single PP2A-A gene—

MpPP2A-A—in its genome, making it an ideal system for in-depth functional studies of PP2A-A.

The CRISPR/Cas9 system is a widely used gene editing tool that has advanced plant biology research and crop improvement. Its simplicity and high efficiency make it a powerful approach for studying plant gene function (Huo et al., 2019). CRISPR/Cas9 induces targeted genome mutations through directed cleavage of specific DNA sequences, thereby revealing gene function (Ma and Liu, 2015). This technology has been successfully applied in numerous plants including rice (*Oryza sativa*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*), and sweet orange (*Citrus sinensis*) (Zeng and Hou, 2015), demonstrating its broad potential and practical value in plant genome editing. Although CRISPR/Cas9 research in *M. polymorpha* remains relatively limited compared with other plants, highly efficient CRISPR/Cas9 vectors for *M. polymorpha* genome editing have been developed (Sugano et al., 2018), enabling functional investigation of MpPP2A-A through gene editing.

This study employed bioinformatics, real-time quantitative PCR, and CRISPR/Cas9 technology in the emerging model plant *M. polymorpha* to address the following questions: (1) Physicochemical properties and structural analysis of MpPP2A-A protein; (2) Phylogenetic relationships of MpPP2A-A and conservation analysis of PP2A-A; (3) Promoter activity prediction of MpPP2A-A; (4) Tissue-specific expression analysis of MpPP2A-A in *M. polymorpha*; (5) Phenotypic analysis of MpPP2A-A knockout mutants. Through preliminary investigation of MpPP2A-A, this study aims to elucidate the fundamental functions of PP2A-A and establish a foundation for further exploration of the molecular mechanisms underlying PP2A regulation of plant growth and development.

Materials and Methods

1.1 Materials

Wild-type male *M. polymorpha* Tak1 was provided by the Hunan Engineering Research Center for Lilium Germplasm Innovation and Deep Processing at Hunan University of Technology. RNA extraction kits, first-strand cDNA synthesis kits, and qPCR-PreMix were purchased from Nanjing Vazyme Biotech Co., Ltd. and Roche GmbH, respectively. Agarose and nucleic acid dyes were obtained from Sangon Biotech (Shanghai) Co., Ltd. Gateway™ LR Clonase™ Enzyme mix was purchased from Thermo Fisher Scientific.

1.2 Primer Design

Primers were designed using SnapGene software. The cloning primers for MpPP2A-A were MpPP2A-CDS-F/MpPP2A-CDS-R; quantitative PCR

primers were MpPP2A-qPCR-F/MpPP2A-qPCR-R; reference gene primers were MpAPT3-qPCR-F/MpAPT3-qPCR-R; sgRNA sequences for knockout vector construction were Oligo1 and Oligo2; primers for positive clone identification were MpPP2A-crispr-1-F/M13R; primers for transgenic plant PCR identification and sequencing were Cas9-F/Cas9-R and MpPP2A-crispr-12-jc-F/MpPP2A-crispr-12-jc-R. Detailed primer sequences are provided in Table 1 .

Table 1 Sequences of primers used in this experiment

Primer name	Sequence (5'–3')
MpPP2A-CDS-F	GCCGCCCCCTTCACCATGGCCATGGTAGACGAGCC
MpPP2A-CDS-R	GCCCTTGCTCACCATCGCGCACATGACTTGCTCG
MpPP2A-qPCR-F	CATGCAGTGGCTTGGTGATAAG
MpPP2A-qPCR-R	ACCAAATTCTTCGGCAAGCC
MpPP2A-crispr-1-F	GCACCCAGCCTCTCGCCGTTGGAGACTCTGTGTACGTTTTAGAGC
MpPP2A-crispr-12-jc-F	ATGGCCATGGTAGACGAGCC
MpPP2A-crispr-12-jc-R	CTTGACCAAGGGAATGAACCAGTCG
MpAPT3-qPCR-F	CGAAAGCCCAAGAAGCTACC
MpAPT3-qPCR-R	GTACCCCCGGTTGCAATAAG
Cas9-F	CAGGCAGATCACTAAGCACGTTG
Cas9-R	AGCGAAATCCCTTCCCTTATCCC
Oligo1	GCACCCAGCCTCTCGGTACACAGAGTCTCCAACGGGTTTTAGAGC
Oligo2	GCACCCAGCCTCTCGGTACACAGAGTCTCCAACGGGTTTTAGAGC

1.3.1 Gene Cloning

Male *M. polymorpha* Tak1 plants are genetically relatively stable and have been widely used in thallus transformation studies. Therefore, this study utilized Tak1 as experimental material. Total RNA was extracted using a plant total RNA extraction kit from Nanjing Vazyme Biotech Co., Ltd. The RNA was reverse-transcribed into cDNA using a reverse transcription kit, and the MpPP2A-A coding sequence was amplified by PCR using high-fidelity polymerase from Takara.

1.3.2 Bioinformatics Analysis

Physicochemical properties of MpPP2A-A protein were analyzed using ProtParam (https://web.expasy.org/protparam/). Hydrophobicity was assessed using ProtScale (https://web.expasy.org/protscale/). Secondary structure was predicted using SOMPA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_{automat}.pl?page=/NPSA/npsa_{sopma}.html). Tertiary structure modeling was performed using Swiss Model (https://swissmodel.expasy.org/). Transmembrane domains were predicted using TMHMM (https://services.healthtech.dtu.dk/services/TMHMM 2.0/). Signal peptides were identified using SignalP (https://services.healthtech.dtu.dk/service.php?SignalP). Functional domains were predicted using SMART (http://smart.embl.de/).

A nucleotide phylogenetic tree was constructed using MEGA 11. Promoter activity analysis was conducted using PlantCARE and visualized with TBtools.

1.3.3 Expression Analysis of MpPP2A-A

Approximately 20-day-old wild-type *M. polymorpha* Tak1 plants were harvested, and three tissues—apical notch, gemma cup, and thallus—were collected separately. RNA was extracted from each tissue following the plant RNA extraction kit protocol. RNA quality and integrity were verified by electrophoresis, and cDNA was synthesized according to the first-strand cDNA synthesis kit instructions. The MpAPT3 gene, which shows stable expression across *M. polymorpha* tissues, served as the internal reference. Real-time quantitative PCR was performed using Roche fluorescent quantitative PCR reagents to analyze MpPP2A-A expression levels in different tissues.

1.3.4 Construction of MpPP2A-A Knockout Mutants

Following CRISPR/Cas9 design principles, a target sequence near the start codon ATG was selected using the CRISPR design website (http://crispr.hzau.edu.cn/CRISPR2/). A single sgRNA sequence targeting Exon 1 of MpPP2A-A was designed: GTACACAGAGTCTCCAACGG (PAM: CGG). Adapters were added to the sense and antisense strands for vector construction. The target sequences were Oligo1 (5 –gcacccagcctctcgGTACACAGAGTCTCCAACGGgttttagagctagaa–3) and Oligo2 (5 –ttctagctctaaaacCCGTTGGAGACTCTGTGTACcgagaggctgggtgc–3), with lowercase letters indicating adapters and uppercase letters indicating target sequences. The two sgRNA oligos were annealed in a reaction containing 10 L Oligo1 (10 μmol · L^{-1}), 10 L Oligo2 (10 μmol · L^{-1}), and 10 L ddH_2O. The mixture was incubated at 96°C for 5 minutes and cooled naturally to room temperature.

The pMpGE-En01 entry vector was digested with PstI and SacI, and the annealed product was ligated to the digested plasmid using the ClonExpress II recombination cloning kit from Nanjing Vazyme Biotech Co., Ltd. The ligation product was transformed into *E. coli* competent cells, plated on LB solid medium containing kanamycin, and incubated overnight at 37°C. Single colonies were selected, verified by colony PCR, cultured, and sequenced by Fuzhou Yashang Biotechnology Co., Ltd. Using Gateway™ LR Clonase™ Enzyme mix, the correctly sequenced entry vector (containing attL sites) was recombined with the destination vector (containing attR sites) *in vitro*. The recombination product was transformed into *E. coli* competent cells, plated on LB solid medium containing spectinomycin, and incubated overnight at 37°C. Single colonies were selected, verified by colony PCR, cultured, and sequenced. After successful sequencing, the plasmid was extracted and transformed into *Agrobacterium* competent cells.

1.3.5 *Marchantia* Transformation, Mutant Identification, and Phenotypic Analysis

Approximately 14-day-old wild-type *M. polymorpha* Tak1 plants were wounded at the thallus margins and pre-cultured for 3 days at 22°C under full light. *Agrobacterium* containing the MpPP2A-A knockout vector was activated simultaneously. After 3 days, the *Agrobacterium* was co-cultured with pre-cultured Tak1 at 22°C, 150 $r \cdot min^{-1}$ under full light for 3 days. The co-cultured *M. polymorpha* was washed with sterile water and plated on selection medium (containing hygromycin and cefotaxime) for approximately 3 weeks to obtain transformants.

DNA was extracted from transformants using the CTAB method. PCR with primers Cas9-F/Cas9-R was used to verify vector integration into the *M. polymorpha* genome. Primers MpPP2A-crispr-jc-12-F/MpPP2A-crispr-jc-12-R were used to amplify the DNA fragment containing the gRNA target site, and PCR products were sequenced by Fuzhou Yashang Biotechnology Co., Ltd. SnapGene was used to align sequencing results with wild-type Tak1 sequences for knockout analysis.

Results

2.1 Full-Length Cloning of MpPP2A-A Coding Region

In *Arabidopsis*, three genes encode PP2A-A subunits: PP2Aa1/RCN1, PP2Aa2, and PP2Aa3 (Locus: At1g25490, At3g25800, and At1g13320). To identify homologous genes in *M. polymorpha*, amino acid sequence alignment was performed on the *Marchantia* website (https://marchantia.info/). A highly similar sequence was identified as the sole homolog, Locus Mp1g29120, designated as MpPP2A-A. Sequence analysis using DNAMAN revealed 91.62% amino acid similarity among the three AtPP2A-A proteins and MpPP2A-A (Figure 1 [Figure 1: see original paper]), indicating functional conservation during evolution.

To obtain the MpPP2A-A coding sequence, total RNA was extracted from ~20-day-old *M. polymorpha* (Figure 2 [Figure 2: see original paper]A) and reverse-transcribed into cDNA. Based on the known MpPP2A-A sequence, CDS primers MpPP2A-CDS-F/MpPP2A-CDS-R were designed. PCR amplification using *M. polymorpha* cDNA as template yielded a DNA fragment of the expected size (Figure 2B). This fragment was successfully cloned into the pENTR vector using seamless ligation technology. PCR identification and sequencing of positive recombinant plasmids confirmed that MpPP2A-A contains a 1,761 bp coding region encoding 586 amino acids, matching the predicted full-length coding sequence.

Figure 1 PP2A-A amino acid sequence alignment between *Arabidopsis thaliana* and *Marchantia polymorpha*

Figure 2 Total RNA extraction and MpPP2A-A gene amplification in *M. polymorpha* Tak1. (A) Electrophoretogram of total RNA extraction; (B) CDS sequence amplification of MpPP2A-A gene (M: DL5000 DNA Marker; 1: Electrophoretic result of MpPP2A-A CDS).

2.2.1 Primary Structure of MpPP2A-A Protein

ProtParam analysis of MpPP2A-A primary structure revealed a molecular formula of $C_{2907}H_{4691}N_{773}O_{852}S_{34}$, isoelectric point (pI) of 4.92 (classifying it as an acidic protein), aliphatic index of 112.15, and instability coefficient of 38.50 (proteins with instability coefficient <40 are considered stable). The protein is thus categorized as stable. It comprises 586 amino acids, including 80 negatively charged residues (Asp+Glu) and 56 positively charged residues (Arg+Lys). Leucine (Leu) accounts for 12.8% of residues, while alanine (Ala) and valine (Val) constitute 9.2% and 8.9%, respectively (Table 2). ProtScale analysis of hydrophilicity/hydrophobicity (with positive values indicating hydrophilicity and negative values hydrophobicity) showed maximum hydrophobicity of 2.356 and maximum hydrophilicity of -3.200 (Figure 3 [Figure 3: see original paper]), indicating strong hydrophobic residues and suggesting that MpPP2A-A is a hydrophobic protein.

Table 2 Amino acid composition of MpPP2A-A protein

Amino acid	Quantity	Proportion (%)	Amino acid	Quantity	Proportion (%)
Ala (A)			Lys (K)		
Arg (R)			Met (M)		
Asn (N)			Phe (F)		
Asp (D)			Pro (P)		
Cys (C)			Ser (S)		
Gln (Q)			Thr (T)		
Glu (E)			Trp (W)		
Gly (G)			Tyr (Y)		
His (H)			Val (V)		
Ile (I)			Pyl (O)		
Leu (L)			Sec (U)		

Figure 3 Hydrophilicity and hydrophobicity analysis of MpPP2A-A protein

2.2.2 Secondary and Tertiary Structures of MpPP2A-A Protein

SOPMA prediction of MpPP2A-A secondary structure revealed that among 586 amino acids, α-helices constitute 75.60%, β-turns 2.39%, random coils 17.75%, and extended strands 4.27% (Figure 4 [Figure 4: see original paper]A), indicating α-helices as the predominant secondary structure element. SWISS MODEL tertiary structure prediction identified MpPP2A-A as a serine/threonine

protein phosphatase 2A 65 kDa regulatory subunit Aα isoform, existing as a monomer without associated ligand molecules (Figure 4B). The predicted structure achieved a GMQE (Global Model Quality Estimate) score of 0.81 and QMEAN (Qualitative Model Energy Analysis) score of 0.80$±$0.05, indicating high model reliability and quality.

Figure 4 Secondary and tertiary structures of MpPP2A-A protein. (A) Secondary structure (blue: α-helix; red: extended strand; green: β-turn; purple: random coil); (B) Tertiary structure.

2.2.3 Domain Analysis of MpPP2A-A Protein

TMHMM 2.0 analysis predicted that the first 500 amino acids are extracellular, with ~90% probability of extracellular localization at position 500 and no transmembrane domains (Figure 5 [Figure 5: see original paper]A), suggesting it may be a secreted protein. SMART and SignalP 6.0 analyses identified three functional domains (Figure 5B): RPT1 (11–233 aa), HEAT (242–272 aa), and HEAT_2 (360–461 aa), with no signal peptide detected (Figure 5C).

Figure 5 Transmembrane structure and functional domains of MpPP2A-A protein. (A) Transmembrane structure prediction; (B) Functional domain prediction; (C) Signal peptide prediction.

2.2.4 Phylogenetic Analysis of MpPP2A-A Gene

To investigate the evolutionary relationship of PP2A-A between *M. polymorpha* and other species, NCBI BLAST was used to search for homologous sequences. Representative plants were selected from bryophytes, algae, ferns, gymnosperms, and angiosperms, including *Arabidopsis thaliana*, *Physcomitrium patens*, *Oryza sativa*, *Lunularia cruciata*, *Ceratopteris richardii*, *Betula platyphylla*, *Setaria viridis*, *Diphasiastrum complanatum*, and *Porphyra crispata*. A PP2A-A nucleotide phylogenetic tree was constructed using MEGA 11 (Figure 6 [Figure 6: see original paper]). The results showed that *M. polymorpha* is closely related to three homologous genes in *P. patens*, and MpPP2A-A exhibits high phylogenetic affinity with the *Arabidopsis* RCN1 gene.

Figure 6 Nucleotide phylogenetic analysis of PP2A-A genes in *Marchantia polymorpha* and other species. Pp: *Physcomitrium patens*; Mp: *Marchantia polymorpha*; Ceric: *Ceratopteris richardii*; AT: *Arabidopsis thaliana*; Luann: *Lunularia cruciata*; BPChr: *Betula platyphylla*; Sevir: *Setaria viridis*; Os: *Oryza sativa*; Dicom: *Diphasiastrum complanatum*; Pum: *Porphyra crispata*.

2.2.5 Promoter Activity Prediction of MpPP2A-A Gene

PlantCARE analysis of the MpPP2A-A promoter region revealed that the 3,000 bp upstream sequence contains numerous core promoter elements (TATA-BOX and CAAT-BOX) as well as auxin-responsive elements, light-responsive elements, and methyl jasmonate (MeJA) response cis-regulatory elements (Figure

7 [Figure 7: see original paper]), suggesting that MpPP2A-A expression may be regulated by multiple environmental factors including light, auxin, and MeJA.

Figure 7 Promoter activity analysis of MpPP2A-A gene

2.3 Tissue-Specific Expression Analysis of MpPP2A-A Gene

Real-time quantitative PCR was used to verify MpPP2A-A expression levels in different *M. polymorpha* tissues. The results showed MpPP2A-A expression in all three tissues examined: apical notch, gemma cup, and thallus (Figure 8 [Figure 8: see original paper]). Expression was highest in the apical notch, moderate in the thallus, and lowest in the gemma cup.

Figure 8 Differential expression of MpPP2A-A gene in different tissues of *M. polymorpha*. Values represent mean ± standard error; ***P<0.001,*** *P<0.0001 (Student's t-test).

2.4 Construction of MpPP2A-A Knockout Mutants

2.4.1 sgRNA Vector Construction The two sgRNA oligos were annealed to form double-stranded DNA and ligated into pMpGE-En01 entry vector digested with PstI and SacI. After transformation into *E. coli* competent cells, single colonies were selected and sequenced, confirming correct vector construction (Figure 9 [Figure 9: see original paper]). Using LR Clonase Enzyme mix, the sgRNA cassette was transferred from pMpGE-En01 (entry vector) to pMpGE010 (expression vector). After transformation and sequencing of single colonies, the correct sequence confirmed successful construction of the MpPP2A-A knockout vector.

Figure 9 Sequencing results of MpPP2A-A knockout vector construction

2.4.2 *Agrobacterium* Transformation and Mutant Acquisition The constructed knockout vector was transformed into *Agrobacterium*. *M. polymorpha* thalli were cultured under full light for 3 days, then co-cultured with activated *Agrobacterium* for 3 days (Figure 10 [Figure 10: see original paper]A). After selection and cultivation, DNA was extracted from 13 transgenic lines using the CTAB method. PCR with primers cas9-F/cas9-R detected a single bright band at the expected 450 bp fragment (Figure 10B), confirming successful integration of the Cas9 expression cassette. PCR using primers MpPP2A-crispr-12-jc-F/MpPP2A-crispr-12-jc-R followed by sequencing revealed mutations at the target site in three transgenic lines (Figure 10C), with the remainder being wild-type. These three mutant lines were designated Mppp2a-a-1-2-1, Mppp2a-a-1-2-3, and Mppp2a-a-1-2-5.

Figure 10 Acquisition and identification of MpPP2A-A transformants. (A) Transformants on selection medium; (B) PCR identification of transformants (M: DL5000 DNA Marker; 1–13: 13 transformants); (C) Sequence alignment chromatograms of target sites.

2.5 Phenotypic Analysis of MpPP2A-A Knockout Mutants

Gemmae are asexual reproductive units of *M. polymorpha* that enable rapid population expansion and maintain genetic stability while enhancing adaptation to diverse environmental conditions. To analyze the effects of MpPP2A-A knockout, gemma growth was examined in Mppp2a-a mutants compared with wild-type Tak1. When gemma cups were fully filled (~22 days), 50 gemmae were collected from each of the wild-type and three mutant lines, and their area was measured using ImageJ. Statistical analysis (Figure 11 [Figure 11: see original paper]A) showed average gemma areas of 0.4367 mm^2 in wild-type Tak1, 0.2443 mm^2 in Mppp2a-a-1-2-1, 0.2298 mm^2 in Mppp2a-a-1-2-3, and 0.2744 mm^2 in Mppp2a-a-1-2-5. All three mutant lines exhibited significantly reduced gemma size compared with wild-type Tak1. Additionally, wild-type *M. polymorpha* gemmae possess two opposite apical meristems forming a notch (apical notch), whereas Mppp2a-a knockout mutants showed increased and irregularly shaped apical notches (Figure 11B), indicating that MpPP2A-A deficiency affects normal growth and development.

Figure 11 Phenotypic analysis of Mppp2a-a mutants. (A) Gemma area statistics for Tak1 and mutants (values: mean ± standard error; ****$P<0.0001$, Student's t-test); (B) Phenotypic comparison of Tak1 and mutants (red arrows indicate apical notch; scale bar: 0.25 mm).

Discussion and Conclusion

Protein phosphatase 2A (PP2A) comprises structural subunit A, regulatory subunit B, and catalytic subunit C. These three subunits are structurally and functionally interdependent, collectively determining PP2A activity and specificity. The catalytic subunit C is the core component responsible for dephosphorylating serine or threonine residues. It forms a core enzyme with subunit A, which then associates with subunit B to form the complete PP2A holoenzyme (Zheng et al., 2020). The structural subunit A provides a scaffold for B and C subunit binding, stabilizing the entire complex. Subunit A plays a key role in regulating specific PP2A activities in cells; mutations or downregulation of its encoding gene cause various cancers in animals and affect plant growth and development. Regulatory subunit B determines PP2A substrate specificity and functional diversity (Morita et al., 2020), with B subunit families (e.g., Bα, Bβ, Bγ) conferring distinct functions and localizations through different variants and combinations. In summary, the three PP2A subunits form highly specific and diverse enzyme complexes that perform critical biological functions in cell proliferation, apoptosis, differentiation, migration, and signal transduction (Seshacharyulu et al., 2013; Nader et al., 2019).

This study focused on the sole PP2A structural subunit A-encoding gene in *M. polymorpha*—MpPP2A-A. Amino acid sequence alignment between *M. polymorpha* and *Arabidopsis* demonstrated that MpPP2A-A is evolutionarily conserved.

Real-time quantitative PCR revealed MpPP2A-A expression in thallus, gemma cup, and apical notch, similar to expression patterns observed for PP2A-A genes in other plants such as *Nicotiana benthamiana*, where two PP2A structural subunit genes are expressed in various tissues (Chen et al., 2018).

Knockout of MpPP2A-A significantly reduced gemma area, consistent with the dwarf phenotype observed in *Arabidopsis* mutants. Previous studies reported that PP2A-A participates in auxin transport pathways in *Arabidopsis* by dephosphorylating PIN auxin transporters, altering their phosphorylation status and distribution, thereby affecting auxin distribution and root development (Michniewicz et al., 2007). Recent research indicates that the PP2A-A subunit can directly bind salicylic acid (SA) to regulate PIN phosphorylation and auxin transport (Tan et al., 2020). Therefore, the reduced gemma size in *M. polymorpha* likely correlates with altered auxin distribution, warranting further investigation. Additionally, Mppp2a-a knockout mutants exhibited early morphological changes. While wild-type Tak1 typically possesses two regularly symmetrical apical notches controlling growth, mutants displayed increased, asymmetric, and irregular notches, suggesting MpPP2A-A involvement in cell division processes.

Beyond plant growth and development, PP2A-A has been implicated in breaking seed dormancy in beech (*Fagus sylvatica*) (González-García et al., 2006) and regulating phototropic responses and blue light-induced stomatal opening (Tseng and Briggs, 2010). Promoter analysis suggests MpPP2A-A may also be associated with light and stress responses. In abiotic stress responses, RCN1 positively regulates plant responses to ionic, osmotic, and oxidative stress (Blakeslee et al., 2008), while maize ZmPP2Aa1 improves low-phosphate tolerance by remodeling root architecture (Wang et al., 2017). These findings underscore the importance of PP2A-A subunits in multiple plant processes and provide valuable insights for future studies in *M. polymorpha*.

In summary, this study identified the sole PP2A-A subunit-encoding gene in the *M. polymorpha* genome, successfully cloned MpPP2A-A, and predicted its physicochemical properties and structural features. Phylogenetic analysis clustered MpPP2A-A with *Arabidopsis* RCN1. Real-time quantitative PCR revealed tissue-specific expression patterns and developmental effects. MpPP2A-A knockout caused significant changes in gemma size and morphology, implicating this gene in regulating gemma growth, apical notch formation, and symmetry maintenance. These results establish a foundation for further exploration of PP2A-A molecular mechanisms in regulating plant growth and provide new insights into *M. polymorpha* developmental biology and environmental adaptation strategies.

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