



國立臺灣大學生物資源暨農學院生物科技研究所

碩士論文

Institute of Biotechnology

College of Bioresources and Agriculture

National Taiwan University

Master's Thesis

利用 *Marchantia polymorpha* 葉狀體轉殖技術研究

高溫影響 miR11707-MpAGO1 之調控功能

Investigation of the regulatory function of miR11707-
MpAGO1 under heat stress using thallus transformation of
Marchantia polymorpha

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中華民國 114 年 7 月

July 2025

國立臺灣大學碩士學位論文

口試委員會審定書

MASTER'S THESIS ACCEPTANCE CERTIFICATE NATIONAL TAIWAN UNIVERSITY

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(系/所/學位學程) 完成之碩士學位論文，於民國 114 年 7 月 25 日承下列
考試委員審查通過及口試及格，特此證明。

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致謝



首先，特別感謝我的指導教授林詩舜老師。從大四至今三年來，老師不僅在我的研究方向上給予了循循善誘的教導，更在論文撰寫的衝刺階段提供了莫大的幫助與寶貴的建議。沒有老師悉心指導，我無法如此順利地完成這本論文。同時，也要感謝邱子珍教授、陳荷明教授、劉力瑜教授以及 Takashi Araki 教授，感謝您們撥冗參與我的碩士論文口試，並給予我許多專業的建議，使我的論文更加完善。此外，也謝謝羅靜琪教授，感謝您在我大學時期的啟蒙與指導，引領我探索科學的世界。

這三年來，我真的很幸運有實驗室的大家以及我的朋友們。感謝育翎學姐一步步帶領我熟悉實驗流程，並耐心解答我所有的疑問；感謝瑄斐學姐分享寶貴的實驗知識；感謝如婷學姐與芊燕學姐在兔房相關問題上給予的協助；感謝昭君學姐以及薇倫學姐一直以來都是實驗室最大的支柱；也感謝芳英學姐、Pookie 學姐以及炳男學長在實驗上的幫助。同時，感謝與我一同奮鬥的同學們昭慈和亮合，我們一起走過了這段不平凡的道路。感謝學妹家齡在我需要時總是給我很大的支持；佳蓁在我遇到電腦或程式碼問題時，她總能提供我協助；還有 Huong Giang、俞如、苓苓一直以來的幫助。此外，也特別感謝筱珺學姐不厭其煩地指導我解決統計以及論文上困難。

最後要感謝我的家人，爸爸、媽媽以及哥哥，你們是我最大的精神支柱。無論是在實驗遇到瓶頸焦頭爛額，或是在寫論文時感到迷惘，你們總是在背後默默地支持我、鼓勵我，給我面對一切的力量。

摘要

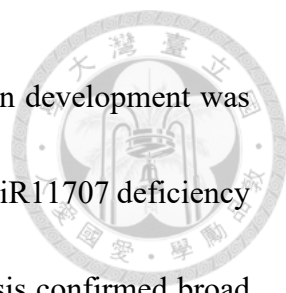
植物微型 RNA (microRNAs; miRNA) 在基因表達調控中扮演關鍵角色。Argonaute (AGO) 蛋白作為核糖核酸誘導沈默複合體 (RNA-induced silencing complex; RISC) 的核心組件，負責介導目標 mRNA 的裂解或轉譯抑制。在早期陸生植物 *Marchantia polymorpha* 中，miR11707.1 以及 miR11707.2 是兩的特有的 miRNA，源自於同一前驅物，並且都能被載入 MpAGO1 中，調控 MpAGO1 mRNA。儘管先前的研究已經為 miR11707-MpAGO1 調控模組提供了基礎見解，但對其功能和分子機制的更詳細理解仍有待深入。本研究利用 Clustered Regularly Interspaced Short Palindromic Repeat-CRISPR associated protein 9 (CRISPR-Cas9) 基因編輯技術結合葉狀體轉殖，在 Takaragaike-1 (Tak-1) 和 Takaragaike-2 (Tak-2) 兩種遺傳背景下成功建立了 *mir11707^{ge}* 突變株，有效降低先前配子體轉殖而產生的後代因染色體重組所引起的遺傳背景變異。降解組分析顯示，在 28°C 下，RISC 活性發生改變，並且不同遺傳背景下 *mir11707^{ge}* 突變株也呈現出 miRNA 目標基因的切割差異。在外表型上，*mir11707^{ge}* 突變體表現出較小的葉狀體和葉狀體邊緣捲曲，其中 *mir11707^{ge}*/Tak-2 在 28°C 下顯示出更高的敏感性。利用遠紅光誘導，高溫抑制了野生型和 *mir11707^{ge}* 突變株的有性生殖器官發育，然而在正常溫度下 miR11707 缺失對有性生殖器官誘導沒有顯著影響。啟動子分析證實 *MIR11707* 在芽孢中廣泛表達，然而 MpAGO1 啟動子活性較低或無法檢測。總而言之，本項研究闡明了 miR11707-MpAGO1 調控在 *M. polymorpha* 中扮演著的關鍵角色，並暗示了遺傳背景可能影響 RNA 沉默，為早期陸生植物在環境壓力下 RNA 沉默途徑的演化和功能提供了新見解。

關鍵字：苔蘚植物，地錢，微型 RNA, Argonaute 1, 葉狀體轉殖

Abstract



Plant microRNAs (miRNAs) play crucial roles in regulating gene expression, with Argonaute (AGO) proteins serving as core components of the RNA-induced silencing complex (RISC), which mediates target mRNA cleavage or translational repression. In the early land plant *Marchantia polymorpha*, miR11707.1 and miR11707.2 are species-specific miRNAs derived from the same precursor, capable of loading into MpAGO1 for MpAGO1 mRNA cleavage. Although previous studies have provided foundational insights into the miR11707-MpAGO1 regulatory module, a more detailed understanding of its functions and molecular mechanisms remains needed. In this study, we employed Clustered Regularly Interspaced Short Palindromic Repeat-CRISPR associated protein 9 (CRISPR-Cas9) gene editing, combined with thallus transformation, to generate *mir11707^{ge}* mutants in two genetic backgrounds, Takaragaike-1 (Tak-1) and Takaragaike-2 (Tak-2), thereby effectively minimizing genetic background variation caused by chromosomal recombination, which was observed in previous sporeling transformation mutant progenies. Degradome analysis revealed altered RISC activity at 28°C, characterized by the loss of miR11707-mediated negative regulation of MpAGO1, leading to differential cleavage efficiencies of various miRNA target genes. Phenotypically, *mir11707^{ge}* mutants displayed smaller thallus size and thallus margin curling, with *mir11707^{ge}*/Tak-2 showing heightened sensitivity at 28°C. Under far-red



light, induction assays showed that at high temperature, sexual organ development was strongly suppressed in both wild-type and *mir11707^{ge}* mutants, and miR11707 deficiency had no significant effect on sexual organ induction. Promoter analysis confirmed broad expression of *MIR11707* in gemmae, while *MpAGO1* promoter activity was low or undetectable. Collectively, this study elucidates the critical role of the miR11707-*MpAGO1* regulatory module in *M. polymorpha* and provides evidence that genetic background may influence RNA silencing, offering new insights into the evolution and functional dynamics of RNA silencing pathways in early land plants under environmental stress.

Keywords: Bryophyte, *Marchantia polymorpha*, MicroRNA, Argonaute 1, Thallus transformation



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Introduction



Plant microRNAs (miRNAs) are a class of small, non-coding RNAs, typically 20 to 24 nucleotides (nt) in length, that play a key role in regulating gene expression. Mature miRNAs are loaded into Argonaute (AGO) proteins, forming the RNA-induced silencing complex (RISC). These miRNAs guide RISC to precisely cleave target mRNAs or inhibit mRNA translation, thereby regulating gene expression and consequently influencing plant growth, development, and environmental adaptation (Chen, 2009; Song et al., 2019; Zhan and Meyers, 2023). As the core protein of RISC, AGO plays a crucial role in its self-regulation mechanism, which is particularly important for plants to adapt to environmental changes. In the context of global warming, the ability of plants to withstand high temperatures has become vital for their survival.

As one of the earliest land plants, the liverwort *Marchantia polymorpha* has had its whole genome fully sequenced, making it an important model organism for studying of plant evolution and gene regulation (Bowman et al., 2016; Bowman et al., 2017). Although the AGO, being the core protein of RISC, has been identified, the RNA silencing mechanism of *M. polymorpha* still remains in the preliminary exploration stage. *M. polymorpha* has four AGO families: MpAGO1, MpAGO4a, MpAGO4b, and MpAGO9 (Lin and Bowman, 2018). Among them, only MpAGO1 is associated with miRNAs and

involved in regulating RNA silencing, while the other AGOs are believed to participate in RNA-directed DNA methylation primarily (Lin and Bowman, 2018; Hong et al., 2024).

The CRISPR/Cas9 system has become a mainstream and efficient gene editing technology, and a simple, rapid, and effective gene editing has been established in *M. polymorpha* (Sugano et al., 2018). Our laboratory also has extensive experience and expertise in using the CRISPR/Cas9 system to generate various miRNA knockout mutants (Hong et al., 2024; Hung et al., 2024). Hong et al. (2024) demonstrated that knocked out the MpAGO1 gene using gene deletion technology, but only obtained short in-frame fragment mutations of MpAGO1 and were unable to generate complete knockout mutants. This suggests that MpAGO1 plays a critical role in the survival of haploid *M. polymorpha*.

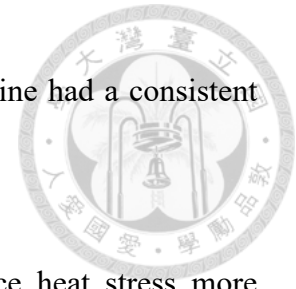
Interestingly, *Arabidopsis thaliana* AGO1 (AtAGO1) is regulated by miR168 (Vaucheret et al., 2004; Vazquez et al., 2004). The non-vascular plant *Physcomitrella patens* has been shown to involve miR904 in the regulation of three PpAGO1 homologues (Arif et al., 2013). *M. polymorpha* confirmed miRNAs, among which miR11707.1 and miR11707.2 processed from the precursor miR11707, both participate in the regulation of MpAGO1 (Lin et al., 2016; Tsuzuki et al., 2016; Lin and Bowman, 2018). Importantly, studies have found that once miR168 or miR11707 are produced, they remain in a certain part of the cytoplasm, waiting for the appropriate time to be loaded into AGO1 self-

regulation (Vaucheret et al., 2006; Jagtap and Shivaprasad, 2014; Dalmadi et al., 2021; Wang et al., 2021; Hong et al., 2024).



M. polymorpha is a dioecious liverwort species, which has Takaragaike-1 (Tak-1) as the male and Takaragaike-2 (Tak-2) as the female. More than half of the genome of the parental Tak-1 and Tak-2 is composed of identical sequences (Tomizawa et al., 2023). However, they still exhibit differences such as single-nucleotide polymorphisms (SNPs) and morphological variations (Bowman et al., 2017; Batth et al., 2025). In the past, mutant lines were generated by sporeling transformation. Although this method can produce a large number of mutants, phenotypic variation arises due to chromosomal recombination between Tak-1 and Tak-2 (Ishizaki et al., 2016). Therefore, the *mir11707^{ge}* mutant lines, which were generated by sporeling transformation exhibited various morphological differences, including asymmetric thallus growth, unexpanded lobe thallus, and multiple lobes appearing in the central area (Hong et al., 2024). Hong et al. (2024) also found that under 28°C, *mir11707^{ge}* mutant lines exhibited varying degrees of thallus twisting, excessive cell proliferation, and abnormal differentiation of the dorsal epidermis. These phenotypes have made it difficult to accurately identify and categorize the effects of miR11707 regulation on *MpAGO1*. Overcome the challenge of inconsistencies due to varied degrees of chromosomal recombination caused by sporeling transformation; this

study employed thallus transformation to ensure that each mutant line had a consistent chromosomal background (Kubota et al., 2013).



In addition, global warming has caused plants to experience heat stress more frequently. Therefore, understanding the regulatory mechanisms of plants under high temperature is crucial, and *AGO* regulation mediated by miRNA play a key role in maintaining plant heat tolerance (Lin et al., 2018; Singh et al., 2022; Blagojevic et al., 2024). Additionally, RNA silencing appears to be closely related to the regulation of sexual reproduction in plants. *AtAGO5* and *AtAGO9* have been found to be highly expressed in reproductive cells (Bradamante et al., 2024). A miR168-resistant *AtAGO1* transformant exhibits sterility and aborted siliques (Vaucheret et al., 2004). Heat-responsive miRNAs such as miR160, miR164, miR159, miR167, and miR169 are also involved in the regulation of sexual reproduction (Ding et al., 2020). In *M. polymorpha*, miR11707 is crucial for maintaining the stability of *MpAGO1* and the RISC activity under 28°C (Hong et al., 2024). Early studies also indicated that using far-red light to induce sexual organs, temperatures above 30°C, temporarily inhibit the development of archegoniophores and antheridiophores in *M. polymorpha* (Lloyd and Steinmetz, 1937).

In this study, we successfully generated *mir11707^{ge}* mutants in two genetic backgrounds (*mir11707^{ge}/Tak-1* and *mir11707^{ge}/Tak-2*) through thallus transformation.

This method effectively eliminated phenotypic interference caused by genetic



background differences. Additionally, using the *mir11707^{ge}* mutants and promoter assays, we further confirmed the regulation of *MpAGO1* by miR11707 under 28°C, as well as the tissue-specific expression patterns of *MIR11707* and *MpAGO1*. We also found that Tak-1 and Tak-2 plants differ in their efficiency of regulating RNA silencing of miRNA target mRNAs. In summary, this study enhances our understanding of the miR11707-*MpAGO1* regulatory mechanism and expands our insights into RNA silencing regulation in *M. polymorpha* across various genetic backgrounds and environmental conditions.



Materials & Methods

Plant materials and growth conditions

M. polymorpha accessions Takaragaike-1 (Tak-1) and Takaragaike-2 (Tak-2) were utilized as wild-type controls in this study. All *M. polymorpha* lines in this study were maintained through asexual propagation of gemmae on half-strength Gamborg's B5 medium (Duchefa) with 1% agar (pH 5.6), under a light intensity of 50–60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at temperatures of 22°C and 28°C. For the induction of sexual organs, *M. polymorpha* lines were cultured from gemmae or thallus under white light conditions supplemented with far-red light (30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) at 22°C and 28°C.

CRISPR-Cas9 vector construction

Two identical miR11707 guide RNAs were cloned using primers designed based on Hong et al. (2024). These primers, listed in Table 1, incorporate *BsaI* restriction sites and facilitate the generation of a gRNA-containing DNA fragment. This fragment was then ligated into the entry vector pMpGE_En03 (Sugano et al., 2018). Following this, the gRNA-containing vector was introduced into the destination vector pMpGE010 (Sugano et al., 2018) via an LR reaction (Invitrogen).



Promoter assay vector construction

To construct the MpAGO1 and MIR11707 promoter reporter constructs, we amplified genomic sequences ranging from 5 to 5.5 kb genomic sequences. For MpAGO1, the amplified region was located upstream of its transcription start site, while for MIR11707, it was upstream of its precursor sequence (Hung et al., 2024). These primers, listed in Table 1. These promoter regions were amplified from wild-type Tak-1 gDNA, subsequently ligated into the pENTR1a entry vector, and finally recombined into the pMpGWB115 vector, which features a Citrine-NLS reporter (Ishizaki et al., 2015).

Thallus transformation

Agrobacterium-mediated transformation of *M. polymorpha* was performed using an established thallus transformation method. Initially, thalli from *M. polymorpha* accessions Tak-1 or Tak-2 were excised and cultured for three days on half-strength Gamborg's B5 medium, supplemented with 1% sucrose and 1% agar. Subsequently, the Agrobacterium tumefaciens strain GV2260 was transformed with either the pMpGWB010_MIR11707_gRNA1 or pMpGWB010_MIR11707_gRNA2 binary vectors for CRISPR-Cas9, or the pMpGWB115_MpAGO1 or pMpGWB115_MIR11707 vectors for promoter assays. This transformed Agrobacterium strain was then co-cultivated with *M. polymorpha* thalli in OM51C medium containing 100 μ M

acetosyringone. Co-cultivation was conducted at 22°C with continuous agitation at 100 rpm for 48 hours.



Following co-cultivation, transformants were thoroughly washed five times with sterile water. Regenerating transformants (T1) were then selected on plates containing 12.5 µg/mL hygromycin (Sigma) and 100 µg/mL cefotaxime until gemmae (G1) were produced. The methodology for these procedures was adapted from Ishizaki et al. (2008) and Kubota et al. (2013). All downstream experiments in this study utilized asexual generations derived after the G1 gemmae stage.

Genotyping

For genotyping, genomic DNA was extracted from approximately 10 mg of thallus tissue with 100 µL of DNA extraction buffer (100 mM Tris-HCl, pH 9.5, 1 M KCl, 10 mM EDTA) by TissueLyser II (QIAGEN). The extracted DNA was then subjected for PCR amplification with 5 µM specific forward and reverse primers (detailed in Table 1) and Taq DNA polymerase 2× Master Mix RED (Ampliqon) and amplification with PCR machine (Biometra). After amplification, the PCR products were checked via electrophoresis at 140 volts for 20 minutes.

For evaluation of the gene-edited genomic DNA region, the PCR products from the gene-edited knockout mutants were purified via Exo-SAP reaction (Exonuclease I, 10×

Exo I buffer, Shrimp Alkaline Phosphatase, 10× TAE buffer) and subsequently submitted for Sanger sequencing to determine the nature of the mutations. Sequence alignment and comparison to the reference genome were performed using Vector NTI software, and the resulting mutation data were compiled.

Small RNA library construction and sequencing

RNA extraction was performed from 50-100 mg of thallus tissue using the XENOPURE™ PF-Total RNA Purification Kit (XENOHHELIX). Library construction followed the XENO-LIBRA kit (XENOHHELIX) protocol. Small RNA sequencing was conducted via next-generation sequencing (NGS) technology, cDNA library was performed on an NovaSeq X Plus (Illumina) sequencing platform using a Paired-End 150 bp (PE150) sequencing strategy. Raw reads were trimmed using CLC Genomic Workbench software (Qiagen) to remove adaptors and low-quality sequences. For comparative analysis, small RNA datasets from Hong et al. (2024) and Hung et al. (2024) were incorporated. The expression of target miRNAs was normalized by counting reads per million (RPM) across all samples. To handle zero expression values and avoid infinite or undefined values during logarithmic transformation, we added a pseudo-count of 1 to all raw RPM expression values prior to log₂ transformation. Expression levels were transformed as log₂ (RPM + 1), and log₂ fold changes were calculated using the formula

$\log_2 [(\text{experimental RPM} + 1) / (\text{control RPM} + 1)]$. This approach ensured stable and meaningful fold change calculations.



Degradome library construction and sequencing

Total RNA was extracted from 50-100 mg of 14-day-old thalli using the PF-Total RNA Purification Kit (XENOHHELIX). mRNA was subsequently isolated from the total RNA using the Dynabeads mRNA DIRECT Purification Kit (Thermo Fisher Scientific). To ensure complete removal of ribosomal RNA, the eluted mRNA underwent an additional purification step with Dynabeads. The isolated mRNA was then denatured at 70°C, followed by ligation of a 12 μM 5' adaptor using T4 RNA ligase 1 (New England Biolabs) at 25°C for 2 hours. Excess 5' adaptors were removed by purifying the ligated mRNA with Dynabeads. The purified 5'-ligated RNA was concentrated by precipitation with isopropanol (Honeywell) and Glycoblue (Thermo Fisher Scientific) at -80°C overnight.

Reverse transcription was performed on the precipitated mRNA using a 20 μM 3' adaptor random primer and a 2.6 μM poly(T) primer with the SuperScript IV Reverse Transcriptase kit (Thermo Fisher Scientific). The resulting cDNA library was amplified by PCR for 10–15 cycles using a forward primer and a unique 250 nM reverse primer, each containing a 6-nt index for sequencing identification. PCR amplification was carried

out with the LongAmp Taq 2× Master Mix kit (New England Biolabs). The amplified cDNA library was purified using Agencourt AMPure XP beads (Beckman Coulter). cDNA concentration was measured using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), and fragment size distribution was assessed with QSep1 (Bioptic).

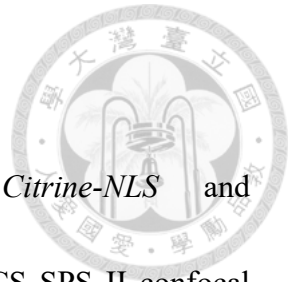
Finally, the cDNA library was sequenced on a NovaSeq X Plus (Illumina) platform using a Paired-End 150 bp (PE150) sequencing strategy. Raw reads were trimmed using CLC Genomic Workbench software (Qiagen) to remove adaptors and low-quality sequences. Expression levels of target miRNAs were normalized by calculating reads per million (RPM) across all samples.

Real-time RT-PCR

Total RNA was extracted from thalli using the Plant Total RNA Extraction Miniprep Kit (Viogene-Biotek Corporation). A total of 2000 ng of extracted RNA was reverse transcribed into cDNA using SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific) with a mixture of random primers and oligo dT20 primers. Quantitative PCR was performed on the LightCycler 480 System (Roche) using the KAPA SYBR FAST qPCR Master Mix (2×) Kit (Sigma-Aldrich). MpEF1-alpha was used as the internal reference gene. All primers used in this study are listed in Table 1.

Microscopic observation of Citrine-NLS signal

To monitor citrine fluorescence in *MIR11707_{pro}:Citrine-NLS* and *MpAGO1_{pro}:Citrine-NLS*, samples were analyzed using a Leica TCS SPS II confocal laser-scanning microscope (Joint Center for Instruments and Instruments and Research, College of Bioresources and Agriculture, National Taiwan University). Citrine fluorescence was detected using a 514 nm laser line for excitation and an emission range of 525-564 nm (PMT2 gain = 525).



Result

CRISPR-Cas9-mediated creation of *mir11707^{ge}* mutants in Tak-1 and Tak-2

backgrounds

To exclude the possibility that phenotypic differences observed in sporeling transformation were caused by sequence variations in the *MIR11707* gene, we first compared the *MIR11707* sequences in chromosomes of Tak-1 and Tak-2 chromosomal backgrounds. The results showed 100% identity between the two sequences (Fig.1 A). Next, we utilized two different guide RNAs (gRNAs) previously designed by Hong et al. (2024): gRNA1 targets the mature strand sequence of miR11707.1, while gRNA2 targets the complementary strand sequence of miR11707.2 (Fig. 1B).

Using thallus transformation, we successfully generated *mir11707^{ge}* mutants in different genetic backgrounds of Tak-1 and Tak-2. The mutants in the Tak-1 background (*mir11707^{ge}/Tak-1* mutants) are prefixed with the letter “M”, including M1-17, M2-1, and M2-2 (Fig. 1C & 1D). The mutants in the Tak-2 background (*mir11707^{ge}/Tak-2* mutants) are prefixed with the letter “F”, including F1-8, F2-1, and F2-3 (Fig. 1C & 1E). The first number in each mutant’s name corresponds to the gRNA number used to generate that mutant.

The *mir11707^{ge}/Tak-1* mutant line M1-17 primarily has an 18-nt deletion on the mature strand of miR11707.1 (Fig. 1C & D). Line M2-1 carries a 12-nt deletion on

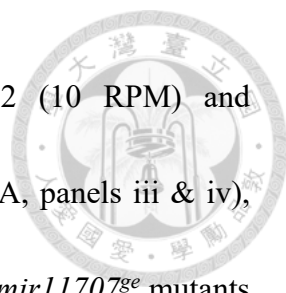


miR11707.1* and miR11707.2*; Line M2-2 exhibits a 71-nt deletion on the mature strands of miR11707.1 and miR11707.2 (Fig. 1C & D). In addition, the *mir11707^{ge}*/Tak-2 mutant line F1-8 has a 17-nt deletion accompanied by a nucleotide substitution of adenine (A) to cytosine (C) on mature strands of miR11707.1 and miR11707.2 (Fig. 1C & E). Line F2-1 contains a 12-nt insertion on the miR11707.1* (Fig. 1C & E), while line F2-3 has a 17-nt deletion on miR11707.1* (Fig. 1C & E).

In summary, we obtained multiple *mir11707^{ge}* mutants, and genotyping results were used for predicted secondary structure analyses, which confirmed that the pre-miR11707 in these mutants exhibited varying degrees of structural alterations. Whether these changes in precursor structure affect the formation of mature miR11707.1 and miR11707.2 still requires further experimental validation.

Small RNA profiling reveals specific miRNA loss and miRNA changes in *mir11707^{ge}* mutants

To confirm the expression levels of miR11707.1 and miR11707.2 in *mir11707^{ge}* mutants, we performed small RNA analysis on 44-day-old Tak-1 plants, *mir11707^{ge}*/Tak-1 mutant line M2-1, and *mir11707^{ge}*/Tak-2 mutant line F1-8. Because the detection of miR11707.1 and miR11707.1* in the Tak-1 plant was incomplete, we could not definitively determine whether miR11707.1 and miR11707.1* were successfully



eliminated (Fig. 2A, panels i & ii). Additionally, miR11707.2 (10 RPM) and miR11707.2* (252.1 RPM) were detectable in Tak-1 plants (Fig. 2A, panels iii & iv), whereas neither miR11707.2 nor miR11707.2* were detected in the *mir11707^{ge}* mutants (Fig. 2A, panels iii & iv). Notably, another miRNA located within the pre-miR11707, miR11707.4, was also undetectable in the *mir11707^{ge}* mutants, while it was detected at 165.1 RPM in Tak-1 plants (Fig. 2A, panel v). These results indicate that the *mir11707^{ge}* mutant lines M2-1 and F1-8 have successfully eliminated the function of the *MIR11707* gene, thus preventing miR11707 production.

Although Hong et al. (2024) detected 6598 reads of miR11707.1 in Tak-1 plants, our small RNA analysis only detected 1 read, making it difficult to accurately assess its abundance in our experimental material. We speculate that this discrepancy may be due to differences in the library preparation methods used in these experiments. Both Hong et al. (2024) and Hung et al. (2024) used Geneaid's miRNA isolation kit and Invitrogen's RNA library kit, whereas our experiment employed XenoHelix's small RNA kit. Comparing the three data sets, Tak-1 plants showed over 5 reads for 322 miRNAs (including mature and star strands), with 108 miRNAs commonly detected across all three datasets. Notably, miR11707.1 was included among the 90 miRNAs shared between the small RNA analyses of Hong et al. (2024) and Hung et al. (2024) (Fig. 2B). We therefore hypothesize that different kits may exhibit bias in capturing, ligating, or amplifying

miRNAs, resulting in certain miRNAs, such as miR11707.1, being inaccurately measured in plant samples.



Additionally, to further rule out data differences caused by transformation methods, we compared the small RNA data obtained in this study with two previously published datasets (Fig. 2C). These datasets include *mir11707^{ge}* mutants produced via sporeling transformation (*mir11707^{ge}_sporeling*) (Hong et al., 2024) and *mir390^{ge}* mutants generated using thallus transformation in the Tak-1 genetic background (*mir390^{ge}_thallus*) (Hung et al., 2024). All three datasets used Tak-1 plants as the reference. The log₂ fold change of small RNA was calculated to compare the trends of small RNA variation under different transformations. In this calculation, a value of 1 indicates a two-fold increase in expression, while a value of -1 indicates a two-fold decrease.

By calculating and comparing the differences among the three mutant lines using log₂ fold change, we observed that miR536 consistently showed a significant decrease in the *mir11707^{ge}* mutants obtained through different transformation methods. Specifically, lines M2-1 and F1-8 exhibited log₂ fold changes of -10.14 and -1.9, respectively, while lines F1 and E12 showed log₂ fold changes of -5.13 and -3.49, respectively (Fig. 2C). In contrast, in the *mir390^{ge}* mutants, the log₂ fold changes of miR536 in KO#18 and KO#21 were 0.58 and 0.76, respectively (Fig. 2C). This indicates that miR536 is significantly

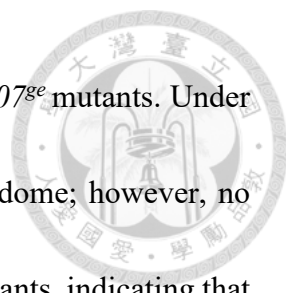
downregulated in *mir11707^{ge}* mutants but shows no significant difference in *mir390^{ge}* mutants.



Therefore, based on the small RNA analysis results, although miR11707.1 was not successfully detected, we clearly confirmed that miR11707.2 and miR11707.4 were successfully eliminated in the *mir11707^{ge}* mutants. This demonstrates that these mutants represent successful knockouts of the *MIR11707* gene. Additionally, data comparison further showed that miR536 expression was significantly reduced in the *mir11707^{ge}* mutants. These findings strongly suggest that knocking out miR11707 may affect the expression of certain small RNAs.

Impact of temperature on RISC-mediated mRNA cleavage and miRNA target regulation in *mir11707^{ge}* mutants

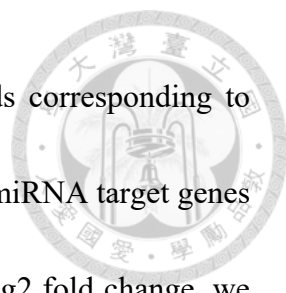
To further confirm whether RISC activity can be promoted at 28°C, we cultivated Tak-1 plants, Tak-2 plants, and *mir11707^{ge}* mutants in different genetic backgrounds, including *mir11707^{ge}*/Tak-1 mutant lines M1-17, M2-1, and M2-2, as well as *mir11707^{ge}*/Tak-2 mutant lines F1-8, F2-1, and F2-3, at both 22°C and 28°C for 14-days-old then assessed the efficiency of miRNA target mRNA cleavage using degradome analysis.



We examined the *MpAGO1* degradome profiles of the *mir11707^{ge}* mutants. Under 22°C, Tak-1 plants showed 0.031 RPM of *MpAGO1* mRNA degradome; however, no degradome signals were detected in any of the *mir11707^{ge}/Tak-1* mutants, indicating that *MpAGO1* mRNA might not have been cleaved (Fig. 3A). In the degradome of Tak-2 plants, 0.152 RPM of *MpAGO1* mRNA degradome was observed. In the *mir11707^{ge}/Tak-2* mutants, except for line F2-1, which showed 0.050 RPM of *MpAGO1* mRNA degradome, all other mutants showed no detectable *MpAGO1* mRNA degradome (Fig. 3B). These results suggest that most *mir11707^{ge}* mutants, having lost miR11707.1, are unable to cleave the target *MpAGO1* mRNA. The exception is line F2-1, where the detected 0.05 RPM *MpAGO1* mRNA degradome is likely due to the nucleotide insertion mutation not completely disrupting the formation of miR11707.1, as predicted by the secondary structure analysis (Fig. 1C).

At 28°C, like those at 22°C, Tak-1 plants were detected with 0.034 RPM of *MpAGO1* mRNA degradome, while Tak-2 plants had 0.13 RPM of *MpAGO1* mRNA degradome. The other *mir11707^{ge}* mutants showed no detectable degradome (Fig. 3A), except for line F2-1, which showed 0.068 RPM of *MpAGO1* mRNA degradome (Fig. 3B).

The 34 degradome reads corresponding to miRNA target genes were obtained from previous studies (Hong et al., 2024). To examine how different genetic backgrounds



affect RISC activity, we additionally analyzed 34 degradome reads corresponding to miRNA target genes. First, we compared the degradome profiles of miRNA target genes between wild-type Tak-1 and Tak-2 plants grown at 22°C. Using log2 fold change, we calculated the changes in miRNA target gene degradomes, taking the Tak-1 plants at 22°C as the reference. This allowed us to assess whether the RISC cleavage efficiency in the Tak-2 plants at 22°C increased or decreased. The results showed that, compared to Tak-1 plants, 26 miRNA target gene degradomes in Tak-2 plants exhibited an increase (Fig. 4A & C). Among these, 5 target genes showed more than a twofold increase in RISC cleavage efficiency, including Mp1g07070.1 (miR160 target), Mp8g12030.1 (miR529c target), Mp7g09870.1 (miR11666.2 target), Mp6g03510.1 (miR11701.2 target), and Mp2g06620.1 (miR11716.1 target) (Fig. 4A & C). Conversely, 6 miRNA target genes in Tak-2 showed decreased RISC cleavage efficiency (Fig. 4B & C). This confirms that Tak-2 plants have higher RISC cleavage efficiency than Tak-1 plants for certain miRNAs.

To compare the RISC cleavage efficiency of *mir11707^{ge}* mutants under different temperature, we analyzed plants from two distinct genetic backgrounds (Tak-1 and Tak-2), each represented by three independent mutant lines. The degradomes of *mir11707^{ge}/Tak-1* mutant lines M1-17, M2-1, and M2-2 were averaged and designated as Tak-1_mutants, while those of *mir11707^{ge}/Tak-2* mutant lines F1-8, F2-1, and F2-3 were averaged and designated as Tak-2_mutants.

Based on the 34 miRNA target genes reported by Hong et al. (2024), we filtered for target genes showing RPM values greater than 0.5 in either Tak-1 or Tak-2 plants at 22°C in our experiment. This filtering resulted in a subset of 20 target genes, which served as the focus of our analysis. The degradome data for all 34 miRNA target genes is provided in Supplementary Fig. 1.

To clarify whether knockout of miR11707 affects RISC cleavage efficiency, we compared the miRNA target gene degradome of Tak-1 and Tak-2 plants with their respective *mir11707^{ge}* mutants at 22°C. Initially, focusing on the *mir11707^{ge}/Tak-1* mutants relative to Tak-1 plants, we observed that 16 miRNA target genes exhibited increased RISC cleavage efficiency (Fig. 5). Conversely, in the *mir11707^{ge}/Tak-2* mutants compared with Tak-2, only 3 miRNA target genes showed increased cleavage efficiency (Fig. 6).

To further assess the effects under high temperature, we examined the degradome profiles of the same genotypes at 28°C. Concentrating first on the *mir11707^{ge}/Tak-1* mutants relative to Tak-1 plants, the data revealed an increase in RISC cleavage efficiency for 4 miRNA target genes (Fig. 7). In contrast, the *mir11707^{ge}/Tak-2* mutants compared to Tak-2 plants exhibited a greater increase in RISC cleavage efficiency, with 18 target genes exhibiting increased cleavage (Fig. 8).



We also analyzed RISC cleavage efficiency in Tak-1 and Tak-2 plants under different temperature conditions. The results showed 14 miRNA target genes showed increased RISC cleavage efficiency at 28°C relative to 22°C (Fig. 9). Additionally, Tak-2 plants exhibited increased RISC activity in 5 miRNA target genes at 28°C compared to 22°C (Fig. 10).

These findings collectively suggest that the impact of miR11707 knockout on RISC cleavage efficiency varies with genetic background and temperature.

Impaction of genetic background and high temperature on thallus size and morphology in *mir11707^{ge}* mutants

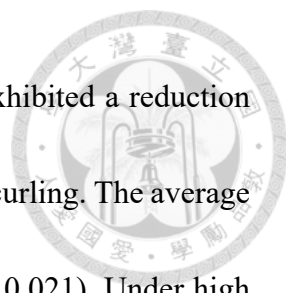
Previous studies have used sporeling transformation to generate *mir11707^{ge}* mutants (Hong et al., 2024). Because these mutants contained varying degrees of chromosome-specific regions from Tak-1 and Tak-2, their progeny exhibited changes in thallus morphology and size at both 22°C and 28°C. To investigate whether thallus transformation can resolve the issue of parental chromosomal recombination, we treated Tak-1 plants, Tak-2 plants, and new *mir11707^{ge}* mutants at various temperatures and observed the thallus morphology, quantifying the thallus area of 14-day-old plants.

At 22°C, the average thallus area of Tak-1 plants was 0.603 cm² ($\pm$ SE 0.020). In contrast, all three *mir11707^{ge}*/Tak-1 mutants showed a reduction in thallus area compared

to Tak-1 plants. Among them, M1-17 displayed the most significant decrease, with an average thallus area of 0.436 cm² ($\pm$ SE 0.012). M2-1 and M2-2 showed moderate reductions, with average thallus areas of 0.511 cm² ($\pm$ SE 0.022) and 0.533 cm² ($\pm$ SE 0.014), respectively (Fig. 11A & B).

In contrast, the average thallus area of Tak-2 plants at 22°C was 0.995 cm² ($\pm$ SE 0.056). In comparison, the *mir11707^{ge}*/Tak-2 line F1-8 showed a significant reduction, with an average thallus area of 0.699 cm² ($\pm$ SE 0.045). Lines F2-1 and F2-3 had average thallus areas of 0.876 cm² ($\pm$ SE 0.026) and 0.817 cm² ($\pm$ SE 0.036), respectively, which also showed a decreasing trend compared to the wild type but were less severe than line F1-8 (Fig. 11C & D).

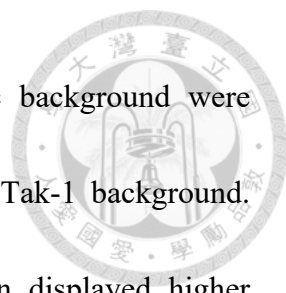
At 28°C, both Tak-1 plants and *mir11707^{ge}*/Tak-1 mutants showed a reduction in average thallus area, accompanied by a distinct phenotype of thallus margin curling. The average thallus area of Tak-1 plants was 0.583 cm² ($\pm$ SE 0.025). Notably, the decrease in thallus area was more pronounced in the *mir11707^{ge}*/Tak-1 mutants at 28°C. Line M1-17 exhibited the most severe reduction, with an average thallus area of only 0.330 cm² ($\pm$ SE 0.013). Lines M2-1 and M2-2 also showed significant decreases in thallus area at 28°C, with average values of 0.495 cm² ($\pm$ SE 0.023) and 0.518 cm² ($\pm$ SE 0.015), respectively (Fig. 11A & B).



Similarly, both Tak-2 plants and *mir11707^{ge}*/Tak-2 mutants exhibited a reduction in average thallus area at 28°C, accompanied by leaf margin upward curling. The average thallus area of wild-type Tak-2 plants decreased to 0.587 cm² ($\pm$ SE 0.021). Under high temperature conditions, all *mir11707^{ge}*/Tak-2 mutants exhibited significant decreases in thallus area. Line F1-8 showed the greatest reduction, with an average thallus area of 0.359 cm² ($\pm$ SE 0.016). Lines F2-1 and F2-3 had average thallus areas of 0.543 cm² ($\pm$ SE 0.034) and 0.556 cm² ($\pm$ SE 0.026), respectively, both significantly lower than the wild type at 28°C (Fig. 11C & D).

To compare the changes in thallus area under high temperature across different genetic backgrounds, we calculated the log₂ fold change at 28°C, using the average thallus area of each line at 22°C as the baseline. The results revealed a more pronounced reduction in thallus area for lines with the Tak-2 background: the Tak-2 plants showed a log₂ fold change of -0.23, while *mir11707^{ge}*/Tak-2 mutant lines F1-8, F2-1, and F2-3 exhibited decreases of -0.29, -0.21, and -0.17, respectively. In contrast, lines carrying the Tak-1 background demonstrated milder changes; the Tak-1 plants had a log₂ fold change of only -0.01, with *mir11707^{ge}*/Tak-1 mutant lines M1-17, M2-1, and M2-2 showing changes of -0.12, -0.01, and -0.01, respectively.

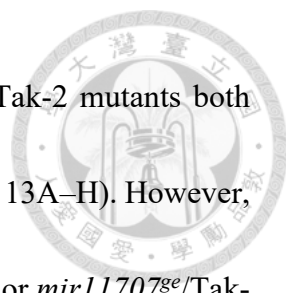
The above phenotypic results show that all *mir11707^{ge}* mutants exhibited reduced thallus area, with the reduction being more pronounced under 28°C, accompanied by



obvious thallus margin curling. Mutants with the Tak-2 genetic background were significantly more sensitive to 28°C compared to those with the Tak-1 background. Interestingly, the mutants obtained through thallus transformation displayed higher consistency and stability in their phenotypes, which helps us observe and evaluate thallus morphological changes in *M. polymorpha* more accurately. These results strongly support the important role of miR11707 in *M. polymorpha* growth, development, and heat stress response.

High temperature suppresses sexual organ formation in both wild-type and *mir11707^{ge}* mutants despite successful induction at 22°C

To investigate whether the *mir11707^{ge}* mutants affect the formation of sexual organs and to understand whether 28°C influences the development of sexual organs in *M. polymorpha*, we induced sexual organ formation in Tak-1 plants, Tak-2 plants, and *mir11707^{ge}* mutants under far-red light. The plants were then observed at 22°C and 28°C for their phenotype. At 22°C, 28-day-old Tak-1 plants and *mir11707^{ge}*/Tak-1 mutants both successfully induced antheridium formation (Fig. 12A–H). However, at 28°C, neither Tak-1 plants nor *mir11707^{ge}*/Tak-1 mutants were able to induce antheridium formation under the same 28-day-old conditions (Fig. 12I–P).



Similarly, at 22°C, 77-day-old Tak-2 plants and *mir11707^{ge}*/Tak-2 mutants both successfully induced archegonium formation under far-red light (Fig. 13A–H). However, under the same 77-day-old conditions at 28°C, neither Tak-2 plants nor *mir11707^{ge}*/Tak-2 mutants were able to induce archegonium formation. These results indicate that *mir11707^{ge}* mutants do not affect the production of sexual organs, revealing that high temperature is the primary factor inhibiting the development of antheridium and archegonium (Fig. 13I–P).

Promoter expression and profiling of *MIR11707* and *MpAGO1* in *M. polymorpha* using a citrine fluorescent reporter

To investigate the specific spatial expression patterns of the *MIR11707* and *MpAGO1* genes in *M. polymorpha*, and to further explore whether there is a correlation between their expression patterns, we used thallus transformation to generate promoter citrine transgenic lines in different genetic backgrounds. By analyzing the fluorescence signals in these transgenic lines, we aimed to determine the distribution patterns of both *MIR11707* and *MpAGO1*.

Confocal microscopy results showed that the citrine fluorescence signal in *MIR11707_{pro}:Citrine-NLS*/Tak-1 transgenic plants was clear and evenly distributed throughout the gemma, while no citrine fluorescence signals were observed in the non-

transgenic Tak-1 control plants (Fig. 14). Similarly, the citrine fluorescence signal in *MIR11707_{pro}:Citrine-NLS/Tak-2* transgenic plants was also evenly distributed in the gemma, and no citrine fluorescence was detected in the non-transgenic Tak-2 control plants (Fig. 15). These results indicate that the *MIR11707* promoter is active in gemmae regardless of whether the genetic background is Tak-1 or Tak-2.

Subsequently, we also performed *MpAGO1_{pro}:Citrine-NLS* construct to assess *MpAGO1* promoter in both Tak-1 and Tak-2 plants. Unexpectedly, we were unable to observe any citrine fluorescence in any of the transgenic lines obtained. This could be due to the *MpAGO1* promoter having very low expression levels, or it might be because the 5'-end of the citrine gene construct we used lacked the 5'-UTR sequence. Even so, qRT-PCR still has the potential to detect citrine expression in *MpAGO1_{pro}:Citrine-NLS* transgenic lines. qRT-PCR results showed that citrine expression was indeed detected in 13 of *MpAGO1_{pro}:Citrine-NLS/Tak-1* transgenic lines (Fig. 16A & C) and 8 of *MpAGO1_{pro}:Citrine-NLS/Tak-2* transgenic lines (Fig. 16B & C), demonstrating that the *MpAGO1* promoter was successfully expressed in the transgenic plants.

Next, we selected *MpAGO1_{pro}:Citrine-NLS* transgenic lines with relatively higher expression levels to analyze the expression of both citrine and endogenous *MpAGO1*. The results showed that the expression levels of citrine in *MpAGO1_{pro}:Citrine-NLS* transgenic lines were similar to those of endogenous *MpAGO1* (Fig. 16D).

MpCYP78A10I_{pro}:Citrine-NLS/Tak-1 plants were used as the experimental control and exhibited citrine expression levels much higher than those of *MpAGO1_{pro}:Citrine-NLS* transgenic lines. In other words, the *MpAGO1* promoter likely has very low expression, which may also explain why no fluorescence signals of *MpAGO1_{pro}:Citrine-NLS* transgenic lines were observed under confocal microscopy.

Discussion

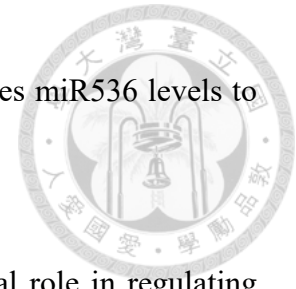
Confirming the regulatory importance of miR11707 for MpAGO1



Due to the limitations of absent biological replicates, inconsistencies in library kits, and technical errors, my results have made it difficult to draw a clear conclusion. To overcome these limitations and establish a robust framework for data interpretation, I referenced results of Hong et al. (2024) to support my data and provide valuable insights into the regulatory functions of miR11707-MpAGO1 in *M. polymorpha*.

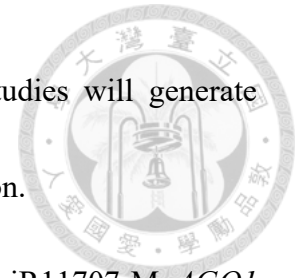
This study provides a more comprehensive understanding of the regulatory function of miR11707-AGO1 in *M. polymorpha* and demonstrates its importance in RNA silencing. In the *mir11707^{ge}* mutants, small RNA-Seq did not detect the sequence of miR11707.2 and miR11707.4 (Fig. 2A). Additionally, the expression level of miR536 was significantly reduced in the *mir11707^{ge}* mutants (Fig. 2C). We also observed that mutations in *MIR11707* affected thallus development, as all *mir11707^{ge}* mutants exhibited reduced thallus area. At 28°C, both wild-type and *mir11707^{ge}* mutants showed even greater reductions in thallus size compared with those at 22°C, often accompanied by thallus curling (Fig. 11). Based on these observations, we infer that the miR11707-MpAGO1 regulatory pathway may influence accumulation of miR536 and modulate growth in *M. polymorpha* through additional downstream mechanisms. Future

experiments will test whether restoring *MIR11707* in mutants rescues miR536 levels to confirm the relationship between both.



Furthermore, our results reveal that miR11707 plays a critical role in regulating RISC activity in *M. polymorpha* under high temperature. Plants are known to respond to heat stress and promote environmental adaptation by modulating the expression of miRNAs and their target genes (Sunkar et al., 2012). Consistent with this, Hong et al. (2024) reported that 28°C promotes the association of miR11707 with MpAGO1 and negatively regulates MpAGO1. Using degradome data at 22°C as a reference, we analyzed RISC activity at 28°C for Tak-1, 65% (13 out of 20) miRNA target genes exhibited increased RISC cleavage efficiency (Fig. 5), corroborating the results of Hong et al. (2024). Similarly, using Tak-1 plants as a reference, we analyzed RISC activity of *mir11707^{ge}*/Tak-1 mutants at 22°C. 65% (13 out of 20) miRNA target genes exhibited increased RISC cleavage efficiency (Fig. 9), also consistent with Hong et al. (2024). Altogether, these results supported the conclusion that high temperature can impact RISC activity in *M. polymorpha*. Under high temperature, RISC activity may be selectively utilized to regulate downstream genes. When miR11707 is knocked out, the slicing efficiency of AGO1 toward its target genes not only increases but may also be indirectly affected or inhibited for certain other specific target genes. This highlights the regulatory complexity of RNA silencing pathways in response to environmental stress. To further

clarify the precise regulatory mechanism of miR11707, future studies will generate transgenic plants that overexpress miR11707 for functional validation.

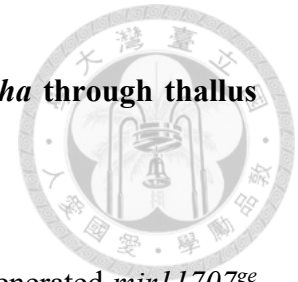


In summary, this study advances our understanding of miR11707-MpAGO1 regulation in *M. polymorpha* and demonstrates the crucial role of miR11707 in the RNA silencing mechanism.

Tissue expression patterns of *MIR11707* and *MpAGO1*

In this study we further investigate the spatial expression patterns of *MIR11707* and *MpAGO1*. The results indicated citrine fluorescence signals in *MIR11707_{pro}:Citrine-NLS* transgenic plants were evenly distributed throughout the gemma. By contrast, we also successfully generated *MpAGO1_{pro}:Citrine-NLS* transgenic plants, but without any citrine fluorescence being detected in these lines. This lack of fluorescence might result either from inherently low promoter activity of *MpAGO1*, or from the design of our reporter construct, which did not include the 5'-UTR sequence of *MpAGO1*. Absence of the 5'-UTR may impair proper protein translation, thereby reducing or abolishing citrine expression. Similar findings, where the absence of a 5'-UTR affects reporter gene expression, have also been reported in other studies (Romani et al., 2024).

Improving the accuracy of phenotypic analysis in *M. polymorpha* through thallus transformation



Compared to the method used by Hong et al. (2024), which generated *mir11707^{ge}* mutants via sporeling transformation and thus resulted in mixed genetic backgrounds due to chromosomal recombination, this method often led to phenotypic inconsistencies, such as asymmetrical thallus growth, incomplete expansion, and the formation of multiple thalli. In this study, we employed thallus transformation, which avoids the parental chromosomal recombination that occurs during sporeling transformation. By generating mutants directly in the Tak-1 and Tak-2 parental we effectively eliminated the influence of chromosomal recombination on the phenotype of the offspring. We confirmed that our *mir11707^{ge}/Tak-1* and *mir11707^{ge}/Tak-2* mutants, at 22°C, exhibited reduced thallus area compared to their respective parental lines. Importantly, these mutants did not display phenotypes such as asymmetrical thalli or the formation of multiple thalli, clearly demonstrating a high level of phenotypic consistency across different lines (Fig. 11). However, it should be noted that differences in transgene insertion sites may still exist during the transformation process (Kubota et al., 2013; Zhu, 2021). Any potential phenotypic effects from these small variations will need to be further examined through multigenerational stability tests and whole-genome analysis. In summary, thallus transformation not only avoids interference from mixed genetic backgrounds but also

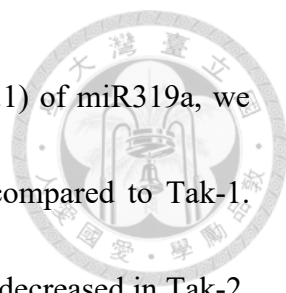
enables us to focus more precisely on investigating the regulatory roles of miR11707-MpAGO1 in environmental adaptation and RNA silencing mechanisms.



Response to high temperature varies across different genetic backgrounds

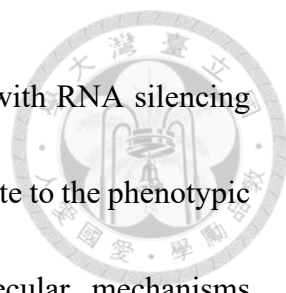
This study highlights how genetic background (Tak-1 and Tak-2) influences the response of *M. polymorpha* to high temperature at both the molecular and phenotypic levels. Our degradome analysis revealed that, even at 22°C, Tak-2 generally exhibits stronger RISC activity than Tak-1, with about 76% (26 out of 34) of miRNA target genes showing increased cleavage efficiency. This indicates differences in RNA silencing activity between the two genetic backgrounds.

Notably, our analysis focused on the 20 miRNA target genes with RPM values above 0.5 at 22°C. At 28°C, 70% (14 out of 20) of miRNA target genes in Tak-1 showed increased RISC activity compared to 22°C, indicating that high temperatures significantly enhance RISC activity and gene silencing. In contrast, only 25% (5 out of 20) of miRNA targets in Tak-2 showed increased activity at 28°C compared to 22°C, indicating a less pronounced response in Tak-2. Moreover, phenotypic observations showed curled thallus margins in both Tak-1 and Tak-2 at 28°C, with a stronger effect in Tak-2, although these morphological changes did not clearly correlate with RISC activity differences.



Interestingly, regarding the target gene *MpRKD* (Mp3g04030.1) of *miR319a*, we observed that RISC activity at 22°C was increased in the Tak-2 compared to Tak-1. However, at 28°C, RISC activity in Tak-1 was increased, whereas it decreased in Tak-2. According to previous studies, *MIR319a* and its target *MpRKD* play key roles in gemma cup and gemma development (Futagami et al., 2025). We speculate that genetic backgrounds may affect the cleavage efficiency of *miR319a* on *MpRKD* under high temperature, thereby influencing the development and adaptability of *M. polymorpha*. This may explain the differing degrees of thallus curling observed between Tak-1 and Tak-2 at 28°C, although the correlation with overall RISC activity changes remains unclear and warrants further investigation. Future studies will focus on determining whether the morphological changes observed under high temperature are directly linked to the regulation by miRNAs and their target genes, using mutants and transgenic plants in both Tak-1 and Tak-2 backgrounds.

In summary, genetic background influences miRNA targets that exhibit changes in RISC activity under high temperature. At 22°C, Tak-2 showed a greater increase in RISC activity compared to Tak-1. At 28°C, targets in Tak-1 led to increased RISC activity, whereas Tak-2 displayed less pronounced changes. However, at 28°C, both Tak-1 and Tak-2 exhibited curled thallus margins, which were more pronounced in Tak-2, without a clear correlation to RISC activity. These findings suggest that genetic background may

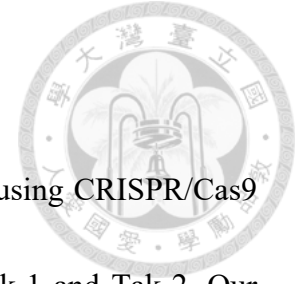


modulate plant responses to high temperature through interactions with RNA silencing pathways, but additional factors beyond RISC activity likely contribute to the phenotypic outcomes. Future studies are warranted to elucidate the molecular mechanisms underlying these regulatory differences and how they influence high temperature adaptation and RNA silencing dynamics across genetic backgrounds.

Effect of high temperature on sexual organs formation

Our study demonstrates that high temperature inhibits the induction of sexual organs in *M. polymorpha*. We found that at 28°C, neither wild type nor *mir11707^{ge}* mutants produced any sexual organs (Fig. 12 & 13). In angiosperms, high temperature has also been shown to affect key steps in sexual reproduction, such as flowering and gametophyte development (Qian et al., 2025). At 22°C, both wild type and *mir11707^{ge}* mutants could be successfully induced to form sexual organs (Fig. 12 & 13). At present, there is no evidence indicating that miR11707 directly affects the induction or formation of sexual organs. The mechanism by which high temperature suppresses sexual reproduction in *M. polymorpha* remains to be elucidated in future studies. Future experiments could perform transcriptome and miRNA profiling of sexual organs to identify heat-responsive genes involved in suppressing sexual reproduction.

Conclusion



This study established *mir11707^{ge}* mutant in *M. polymorpha* using CRISPR/Cas9 and thallus transformation in two distinct genetic backgrounds, Tak-1 and Tak-2. Our findings demonstrate that the miR11707-MpAGO1 regulatory module plays a critical role in RNA silencing, influencing thallus development and heat stress responses. Notably, *mir11707^{ge}* mutants in the Tak-2 background showed greater sensitivity to high temperature. Additionally, the high temperature broadly inhibited sexual organ formation in both wild-type and *mir11707^{ge}* mutant plants independently of miR11707. Future research, including the overexpression lines of *MIR11707*, might provide more detailed studies in RNA silencing pathways in *M. polymorpha* under environmental stress.



References

Arif, M.A., Frank, W., and Khraiwesh, B. (2013). Role of RNA interference (RNAi) in the Moss *Physcomitrella patens*. *Int J Mol Sci* **14**, 1516-1540.

Batth, R., Poormassalehgoo, A., Bhardwaj, K., Kaniecka, E., and Goto-Yamada, S. (2025). A Simple and Scalable Chopped-Thallus Transformation Method for *Marchantia polymorpha*. *Plants (Basel)* **14**.

Blagojevic, A., Baldrich, P., Schiaffini, M., Lechner, E., Baumberger, N., Hammann, P., Elmayan, T., Garcia, D., Vaucheret, H., Meyers, B.C., and Genschik, P. (2024). Heat stress promotes *Arabidopsis* AGO1 phase separation and association with stress granule components. *iScience* **27**, 109151.

Bowman, J.L., Araki, T., and Kohchi, T. (2016). *Marchantia*: Past, Present and Future. *Plant Cell Physiol* **57**, 205-209.

Bowman, J.L., Kohchi, T., Yamato, K.T., Jenkins, J., Shu, S., Ishizaki, K., Yamaoka, S., Nishihama, R., Nakamura, Y., Berger, F., Adam, C., Aki, S.S., Althoff, F., Araki, T., Arteaga-Vazquez, M.A., Balasubramanian, S., Barry, K., Bauer, D., Boehm, C.R., Briginshaw, L., Caballero-Perez, J., Catarino, B., Chen, F., Chiyoda, S., Chovatia, M., Davies, K.M., Delmans, M., Demura, T., Dierschke, T., Dolan, L., Dorantes-Acosta, A.E., Eklund, D.M., Florent, S.N., Flores-Sandoval, E., Fujiyama, A., Fukuzawa, H., Galik, B., Grimanelli, D.,



Grimwood, J., Grossniklaus, U., Hamada, T., Haseloff, J., Hetherington, A.J.,
Higo, A., Hirakawa, Y., Hundley, H.N., Ikeda, Y., Inoue, K., Inoue, S.I.,
Ishida, S., Jia, Q., Kakita, M., Kanazawa, T., Kawai, Y., Kawashima, T.,
Kennedy, M., Kinose, K., Kinoshita, T., Kohara, Y., Koide, E., Komatsu, K.,
Kopischke, S., Kubo, M., Kyoizuka, J., Lagercrantz, U., Lin, S.S., Lindquist,
E., Lipzen, A.M., Lu, C.W., De Luna, E., Martienssen, R.A., Minamino, N.,
Mizutani, M., Mizutani, M., Mochizuki, N., Monte, I., Mosher, R., Nagasaki,
H., Nakagami, H., Naramoto, S., Nishitani, K., Ohtani, M., Okamoto, T.,
Okumura, M., Phillips, J., Pollak, B., Reinders, A., Rovekamp, M., Sano, R.,
Sawa, S., Schmid, M.W., Shirakawa, M., Solano, R., Spunde, A., Suetsugu,
N., Sugano, S., Sugiyama, A., Sun, R., Suzuki, Y., Takenaka, M., et al. (2017).
Insights into Land Plant Evolution Garnered from the *Marchantia polymorpha*
Genome. *Cell* **171**, 287-304 e215.

Bradamante, G., Nguyen, V.H., Incarbone, M., Meir, Z., Bente, H., Dona, M.,
Lettner, N., Mittelsten Scheid, O., and Gutzat, R. (2024). Two ARGONAUTE
proteins loaded with transposon-derived small RNAs are associated with the
reproductive cell lineage in *Arabidopsis*. *Plant Cell* **36**, 863-880.

Chen, X. (2009). Small RNAs and their roles in plant development. *Annu Rev Cell Dev Biol* **25**, 21-44.



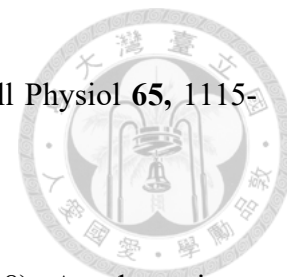
Dalmadi, A., Miloro, F., Balint, J., Varallyay, E., and Havelda, Z. (2021). Controlled RISC loading efficiency of miR168 defined by miRNA duplex structure adjusts ARGONAUTE1 homeostasis. *Nucleic Acids Res* **49**, 12912-12928.

Ding, Y., Huang, L., Jiang, Q., and Zhu, C. (2020). MicroRNAs as Important Regulators of Heat Stress Responses in Plants. *J Agric Food Chem* **68**, 11320-11326.

Futagami, K., Tsuzuki, M., Yoshida, M., and Watanabe, Y. (2025). MpmiR319 promotes gemma/gemma cup formation in the liverwort *Marchantia polymorpha*. *J Exp Bot*.

Hong, S.F., Wei, W.L., Pan, Z.J., Yu, J.Z., Cheng, S., Hung, Y.L., Tjita, V., Wang, H.C., Komatsu, A., Nishihama, R., Kohchi, T., Chen, H.M., Chen, W.C., Lo, J.C., Chiu, Y.H., Yang, H.C., Lu, M.Y., Liu, L.D., and Lin, S.S. (2024). Molecular Insights into MpAGO1 and Its Regulatory miRNA, miR11707, in the High-Temperature Acclimation of *Marchantia polymorpha*. *Plant Cell Physiol* **65**, 1414-1433.

Hung, Y.L., Hong, S.F., Wei, W.L., Cheng, S., Yu, J.Z., Tjita, V., Yong, Q.Y., Nishihama, R., Kohchi, T., Bowman, J.L., Chien, Y.C., Chiu, Y.H., Yang, H.C., Lu, M.J., Pan, Z.J., Wang, C.N., and Lin, S.S. (2024). Dual Regulation of Cytochrome P450 Gene Expression by Two Distinct Small RNAs, a Novel



tasiRNA and miRNA, in *Marchantia polymorpha*. *Plant Cell Physiol* **65**, 1115-1134.

Ishizaki, K., Chiyoda, S., Yamato, K.T., and Kohchi, T. (2008). Agrobacterium-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol* **49**, 1084-1091.

Ishizaki, K., Nishihama, R., Yamato, K.T., and Kohchi, T. (2016). Molecular Genetic Tools and Techniques for *Marchantia polymorpha* Research. *Plant Cell Physiol* **57**, 262-270.

Ishizaki, K., Nishihama, R., Ueda, M., Inoue, K., Ishida, S., Nishimura, Y., Shikanai, T., and Kohchi, T. (2015). Development of Gateway Binary Vector Series with Four Different Selection Markers for the Liverwort *Marchantia polymorpha*. *PLoS One* **10**, e0138876.

Jagtap, S., and Shivaprasad, P.V. (2014). Diversity, expression and mRNA targeting abilities of Argonaute-targeting miRNAs among selected vascular plants. *BMC Genomics* **15**, 1049.

Kubota, A., Ishizaki, K., Hosaka, M., and Kohchi, T. (2013). Efficient Agrobacterium-mediated transformation of the liverwort *Marchantia polymorpha* using regenerating thalli. *Biosci Biotechnol Biochem* **77**, 167-172.

Lin, J.S., Kuo, C.C., Yang, I.C., Tsai, W.A., Shen, Y.H., Lin, C.C., Liang, Y.C., Li,

Y.C., Kuo, Y.W., King, Y.C., Lai, H.M., and Jeng, S.T. (2018). MicroRNA160

Modulates Plant Development and Heat Shock Protein Gene Expression to

Mediate Heat Tolerance in Arabidopsis. *Front Plant Sci* **9**, 68.

Lin, P.C., Lu, C.W., Shen, B.N., Lee, G.Z., Bowman, J.L., Arteaga-Vazquez, M.A.,

Liu, L.Y., Hong, S.F., Lo, C.F., Su, G.M., Kohchi, T., Ishizaki, K., Zachgo, S.,

Althoff, F., Takenaka, M., Yamato, K.T., and Lin, S.S. (2016). Identification

of miRNAs and Their Targets in the Liverwort *Marchantia polymorpha* by

Integrating RNA-Seq and Degradome Analyses. *Plant Cell Physiol* **57**, 339-358.

Lin, S.S., and Bowman, J.L. (2018). MicroRNAs in *Marchantia polymorpha*. *New*

Phytol **220**, 409-416.

Lloyd, C.E., and Steinmetz, F.H. (1937). Temperature as a Factor Influencing the

Sexual Response of *Marchantia*. *American Journal of Botany* **24**, 423-425.

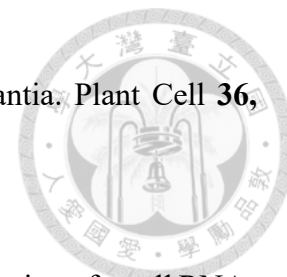
Qian, D., Wang, M., Niu, Y., Yang, Y., and Xiang, Y. (2025). Sexual reproduction in

plants under high temperature and drought stress. *Cell Rep* **44**, 115390.

Romani, F., Sauret-Gueto, S., Rebmann, M., Annese, D., Bonter, I., Tomaselli, M.,

Dierschke, T., Delmans, M., Frangedakis, E., Silvestri, L., Rever, J., Bowman,

J.L., Romani, I., and Haseloff, J. (2024). The landscape of transcription factor



promoter activity during vegetative development in *Marchantia*. *Plant Cell* **36**, 2140-2159.

Singh, R.K., Prasad, A., Maurya, J., and Prasad, M. (2022). Regulation of small RNA-mediated high temperature stress responses in crop plants. *Plant Cell Rep* **41**, 765-773.

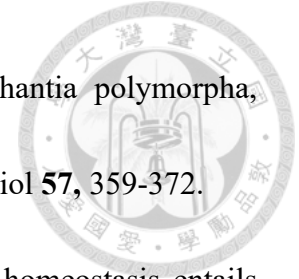
Song, X., Li, Y., Cao, X., and Qi, Y. (2019). MicroRNAs and Their Regulatory Roles in Plant-Environment Interactions. *Annu Rev Plant Biol* **70**, 489-525.

Sugano, S.S., Nishihama, R., Shirakawa, M., Takagi, J., Matsuda, Y., Ishida, S., Shimada, T., Hara-Nishimura, I., Osakabe, K., and Kohchi, T. (2018). Efficient CRISPR/Cas9-based genome editing and its application to conditional genetic analysis in *Marchantia polymorpha*. *PLoS One* **13**, e0205117.

Sunkar, R., Li, Y.F., and Jagadeeswaran, G. (2012). Functions of microRNAs in plant stress responses. *Trends Plant Sci* **17**, 196-203.

Tomizawa, Y., Minamino, N., Shimokawa, E., Kawamura, S., Komatsu, A., Hiwatashi, T., Nishihama, R., Ueda, T., Kohchi, T., and Kondo, Y. (2023). Harnessing Deep Learning to Analyze Cryptic Morphological Variability of *Marchantia polymorpha*. *Plant Cell Physiol* **64**, 1343-1355.

Tsuzuki, M., Nishihama, R., Ishizaki, K., Kurihara, Y., Matsui, M., Bowman, J.L., Kohchi, T., Hamada, T., and Watanabe, Y. (2016). Profiling and



Characterization of Small RNAs in the Liverwort, *Marchantia polymorpha*,
Belonging to the First Diverged Land Plants. *Plant Cell Physiol* **57**, 359-372.

Vaucheret, H., Mallory, A.C., and Bartel, D.P. (2006). AGO1 homeostasis entails
coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by
AGO1. *Mol Cell* **22**, 129-136.

Vaucheret, H., Vazquez, F., Crete, P., and Bartel, D.P. (2004). The action of
ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA
pathway are crucial for plant development. *Genes Dev* **18**, 1187-1197.

Vazquez, F., Gascioli, V., Crete, P., and Vaucheret, H. (2004). The nuclear dsRNA
binding protein HYL1 is required for microRNA accumulation and plant
development, but not posttranscriptional transgene silencing. *Curr Biol* **14**, 346-
351.

**Wang, H., Li, Y., Chern, M., Zhu, Y., Zhang, L.L., Lu, J.H., Li, X.P., Dang, W.Q.,
Ma, X.C., Yang, Z.R., Yao, S.Z., Zhao, Z.X., Fan, J., Huang, Y.Y., Zhang,
J.W., Pu, M., Wang, J., He, M., Li, W.T., Chen, X.W., Wu, X.J., Li, S.G., Li,
P., Li, Y., Ronald, P.C., and Wang, W.M.** (2021). Suppression of rice miR168
improves yield, flowering time and immunity. *Nat Plants* **7**, 129-136.

Zhan, J., and Meyers, B.C. (2023). Plant Small RNAs: Their Biogenesis, Regulatory
Roles, and Functions. *Annu Rev Plant Biol* **74**, 21-51.

Zhu, L. (2021). Targeted Gene Knockouts by Protoplast Transformation in the Moss

Physcomitrella patens. *Front Genome Ed* **3**, 719087.





Table

Table 1. The primer sets employed for gene assembly in this study.

1. CRISPR/Cas9 gene editing gRNA oligonucleotide sequences.

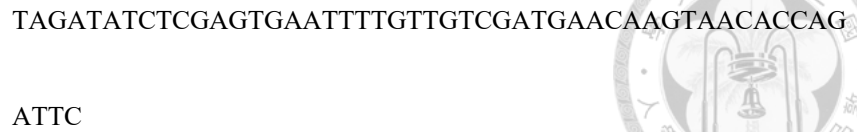
Name	sequence (5' to 3')
miR11707_gRNA1_A	CTCGGGCCAGTTCCCATGGATG
miR11707_gRNA1_B	AAACCATCCATGGGAAGTGGCC
miR11707_gRNA2_A	CTCGTAAGAATGGCCCGAAGAT
miR11707_gRNA2_B	AAACATCTTCGGGCCATTCTTA

2. Primers for genotyping CRISPR/Cas9-edited mutants.

Name	sequence (5' to 3')
miR11707_geno_F	TGGTACAAATGGCACACAATG
miR11707_geno_R	TCCGATCTCTTACAGCGTGTG
pMpGE010_seq_R	CCAGGCTTTACACTTTATGCTTCC

3. Promoter reporters construct oligonucleotide sequences

Name	sequence (5' to 3')
MpAGO1-pro-F	AAGGAACCAATTCAGTCTATTAATCTGATGCTCCCTAACCAAGTAT TAATG
MpAGO1-pro-R	TAGATATCTCGAGTGACTTCCCTTTCAATGCATTCCCCACTT
MpmiR11707-pro-F	AAGGAACCAATTCAGTCCTTCAGGCAATTAGCAGTCG



Name	sequence (5' to 3')
MpAGO1_pro_T1_F	AGGTTTGAACATTCGGTGGAGGGAC
MpAGO1_pro_T2_F	GCATGCATGAGAAAGGAAAGG
MpAGO1_pro_T3_F	AACCTCGAACATCCAGAGCACGA
MpAGO1_pro_T4_F	CAACTCTGTTTTGTTTTCTCG
MpAGO1_pro_T5_F	TCTCCAAAGGCAAACACGCAGAT
MpAGO1_pro_T6_F	CACCCGAACCTTTTGCATGCTCA
MpAGO1_pro_T7_F	TTCTTCTCAGCCGGGTTACG
MpAGO1_pro_T8_R	GAGTCCCTCCACCGAATGTT
MpmiR11707_pro_T1_F	GTATGATGAGAGGAGGAAACACT
MpmiR11707_pro_T2_F	CATGGAAATCAGAACCCGCC
MpmiR11707_pro_T3_F	ATCATCTTGTGCATGGATC
MpmiR11707_pro_T4_F	TGAGCTGGCTAAAGTTCTAT
MpmiR11707_pro_T5_F	CGTGTATTCATGCGGTTGGT
MpmiR11707_pro_T6_F	GAGTCTCGCCTGTTATCCGA



MpmiR11707_pro_T7_R	CCTCCAACAGTGTTCC TCCT
MpmiR11707_pro_T8_R	TCCCTCTGCCATGCCACGAA
pENTR1a_seq	GTAACATCAGAGATTTTGAGACAC

3. Primer sequences for mRNA RTqPCR

Name	sequence (5' to 3')
For-gPCR-MpAGO1	TCCTCTGGTTAGTGACGTTCC
Rev-gPCR-MpAGO1	AATTAGTTCTTGCCGATGTGC
For-gPCR-MpEF1a	AAGCCGTCGAAAAGAAGGAG
Rev-gPCR-MpEF1a	TTCAGGATCGTCCGTTATCC
MpCYP78A_qF_YL	CTCGACTCTCAACGAGCAAG
MpCYP78A_qR_YL	CAGGAATCGGTTTTCTTCCA

Figures

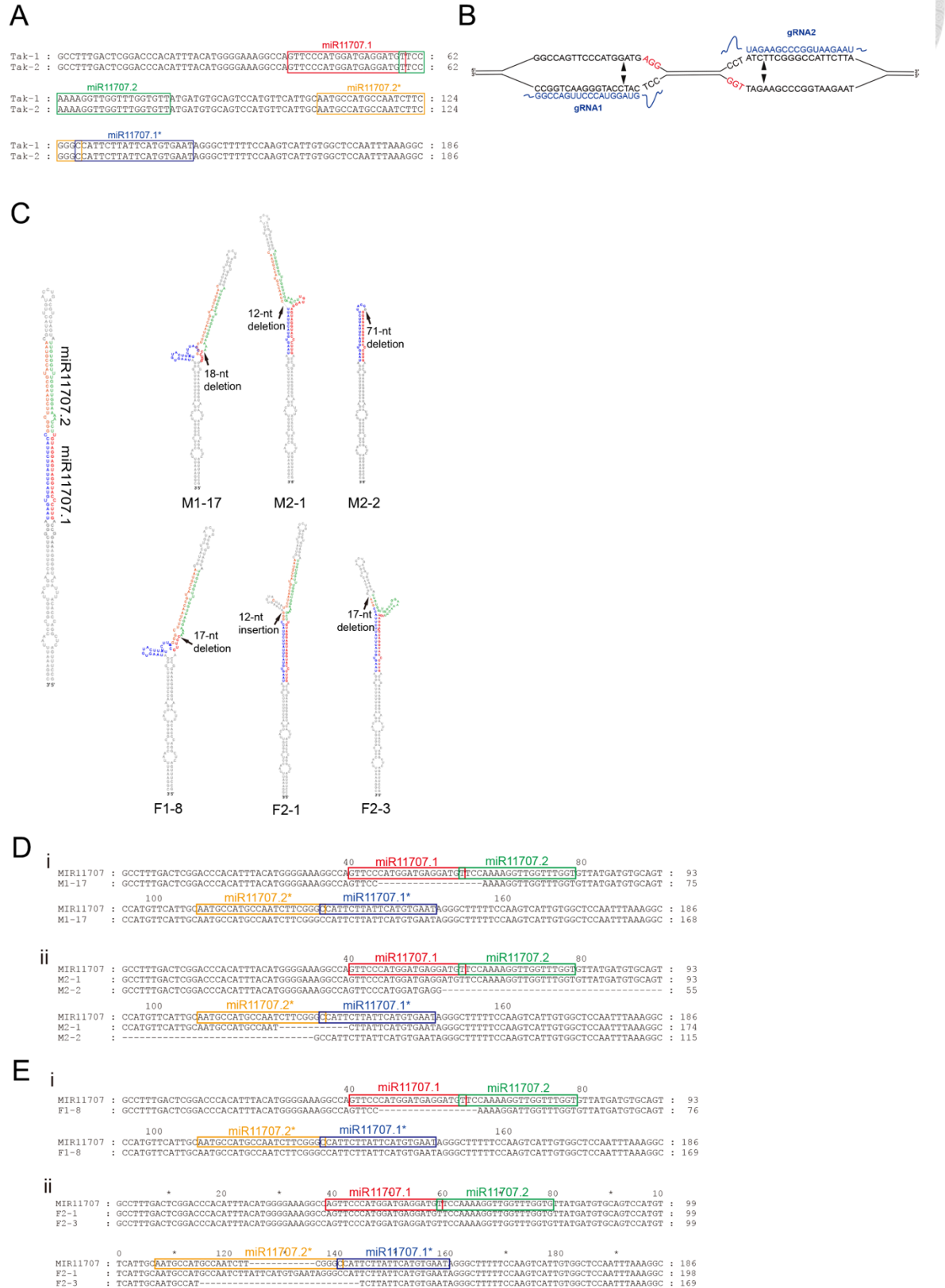


Figure 1. CRISPR-Cas9-mediated mutation of MpMIR11707.



(A) Sequences of the *MIR11707* gene in Tak-1 and Tak-2 backgrounds. Mature miR11707.1 and miR11707.2 are highlighted in red and green, respectively; miR11707.1* and miR11707.2* are shown in orange and blue. (B) Two gRNAs were designed for *MIR11707* gene editing. Red letters indicate the Protospacer Adjacent Motif (PAM) sequence, and the guide RNA sequences are shown in blue. (C) Secondary structure prediction of the wild type and edited *mir11707^{ge}* precursors. The mature and star sequences of miR11707.1 are highlighted in red and blue, respectively, while those of miR11707.2 are highlighted in green and orange, respectively. M: Tak-1 background, F: Tak-2 background. The alignment of the amino acid sequences of the mutants with their references at (i) gRNA1 and (ii) gRNA2, in the (D) Tak-1 and (E) Tak-2 backgrounds, respectively. Mature miR11707.1 and miR11707.2 are highlighted in red and green, respectively; miR11707.1* and miR11707.2* are shown in orange and blue.

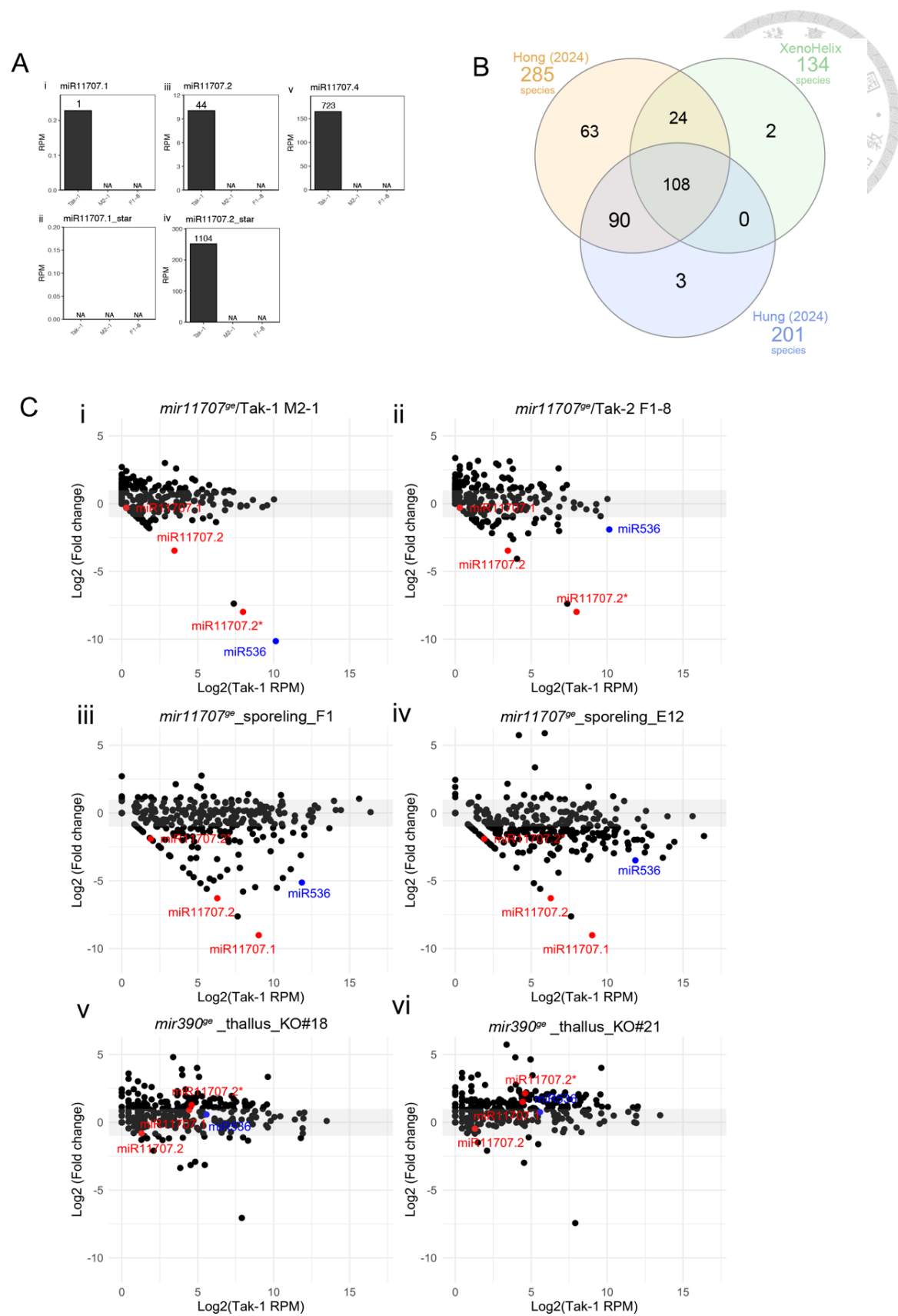
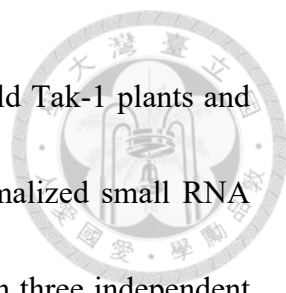


Figure 2. Expression patterns of miR11707 family and related small RNAs in mutant and wild-type plants.



(A) Small RNA analysis of miR11707 family members in 44-day-old Tak-1 plants and *mir11707^{ge}* mutant lines. Reads per million (RPM) represents normalized small RNA expression levels. (B) Venn diagram of miRNA detection patterns in three independent small RNA datasets from Tak-1. XenoHelix represents the small RNA data from this study ; Hong (2024) refers to Hong et al. (2024) small RNA data; and Hung (2024) corresponds to (Hung et al., 2024) small RNA data. (C) Comparative analysis of small RNA log₂ fold changes in *mir11707^{ge}* and *mir390^{ge}* mutants relative to Tak-1 reference plants. *mir11707^{ge}* _sporeling refers to small RNA data from Hong et al. (2024); and *mir390^{ge}* _thallus corresponds to small RNA data from (Hung et al., 2024). MiR11707 family members are shown as red dots, miR536 shown blue dot, and other miRNAs as black dots.

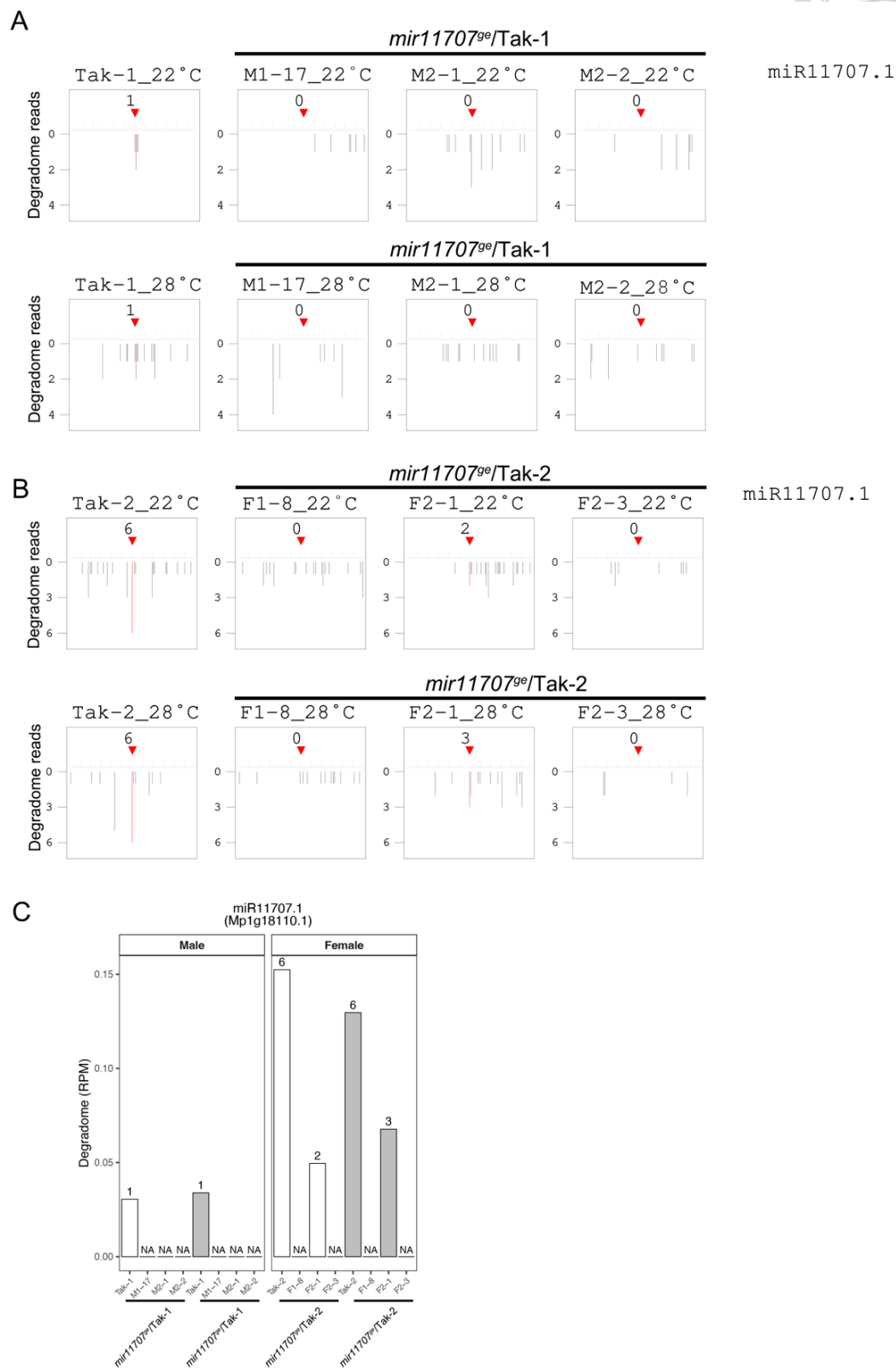


Figure 3. Degradome analysis of MpAGO1 mRNA cleavage by miR11707.1 in Tak-1, Tak-2, and *mir11707^{ge}* mutants under different temperature.

(A) Degradome profiles of MpAGO1 in Tak-1 and *mir11707^{ge}*/Tak-1 mutants. (B) Degradome profiles of MpAGO1 in Tak-2 and *mir11707^{ge}*/Tak-2 mutants at 22°C and 28°C. Red triangles indicate the miR11707.1 cleavage site on MpAGO1 mRNA. Red lines and numbers represent the depth of degradome reads. (C) Bar graph of degradome RPM mapped to MpAGO1 in Tak-1 plants, Tak-2 plants, and *mir11707^{ge}* mutants. The Y-axis represents the RPM of degradome reads. White bars correspond to lines at 22°C, and gray bars correspond to lines at 28°C.

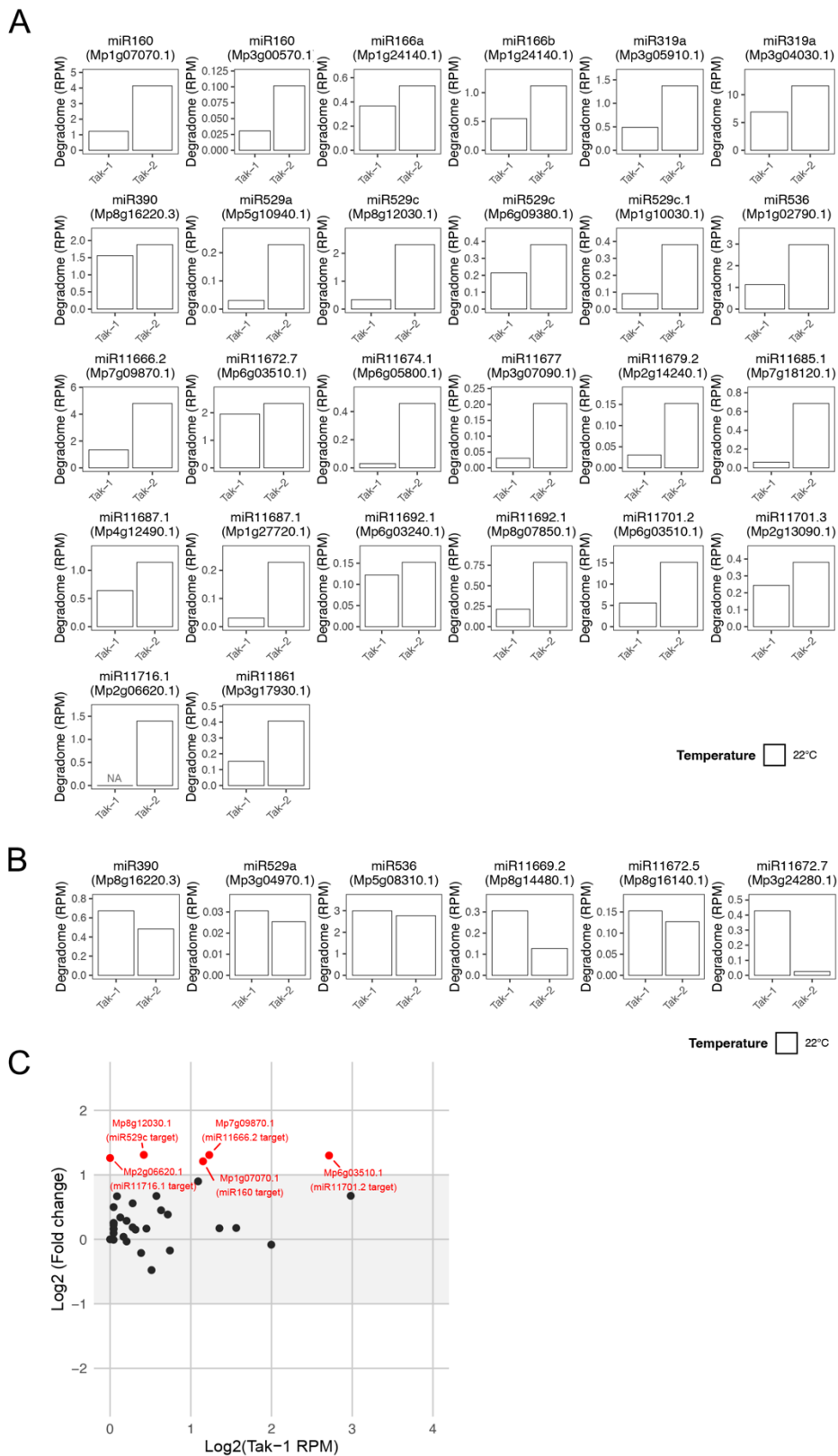
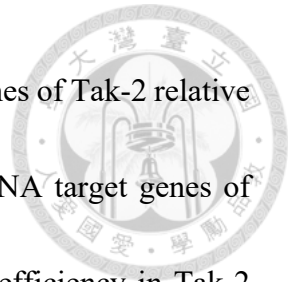


Figure 4. Comparative analysis of RISC cleavage efficiency on miRNA target genes in Tak-1 and Tak-2 at 22°C.

(A) Increased RISC cleavage efficiency in specific miRNA target genes of Tak-2 relative to Tak-1. (B) Decreased RISC cleavage efficiency in specific miRNA target genes of Tak-2 relative to Tak-1. (C) Log2 fold changes of RISC cleavage efficiency in Tak-2 relative to Tak-1. The X-axis represents the log2 of Tak-1 plants RPM. The Y-axis represents the log2 fold change. miRNAs with log2 fold changes greater than 1 are shown as red dots. The gray background spans from -1 to 1 on the Y-axis.



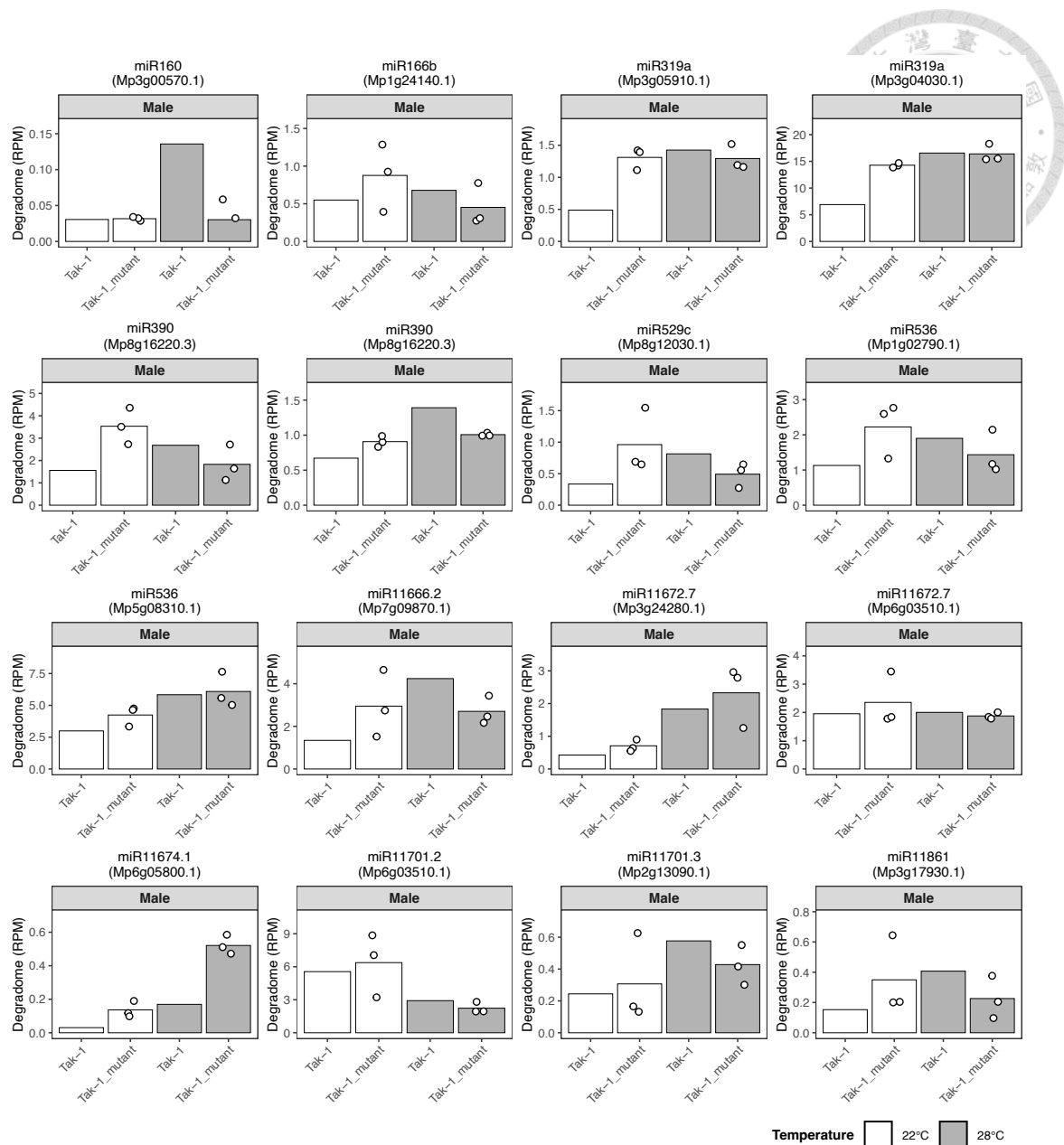


Figure 5. Increased RISC cleavage efficiency in specific miRNA target genes of *mir11707^{ge}/Tak-1* mutants at 22°C.

The increases are relative to their respective wild-type *Tak-1* plants. *Tak-1* mutant represents the *mir11707^{ge}/Tak-1* mutant lines M1-17, M2-1, and M2-2. White bars correspond to data at 22°C, and gray bars correspond to data at 28°C. Each line is shown as a white dot.

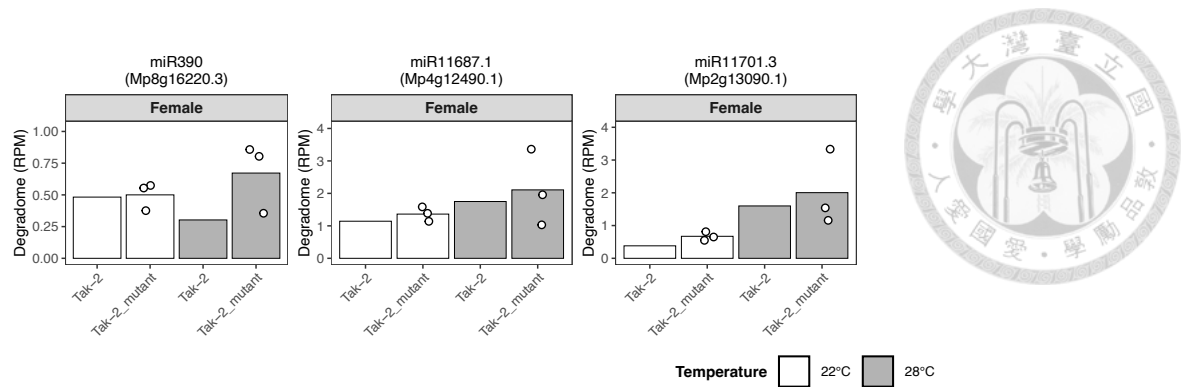


Figure 6. Increased RISC cleavage efficiency in specific miRNA target genes of *mir11707^{ge}/Tak-2* mutants at 22°C.

The increases are relative to their respective wild-type Tak-2 plants. Tak-2_mutant represents the *mir11707^{ge}/Tak-2* mutant lines F1-8, F2-1, and F2-3. White bars correspond to data at 22°C, and gray bars correspond to data at 28°C. Each line is shown as a white dot.

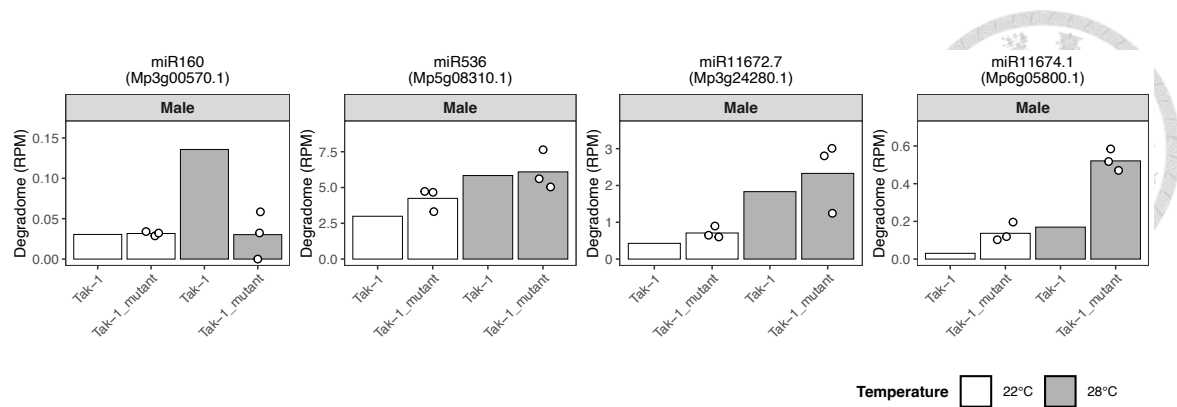


Figure 7. Increased RISC cleavage efficiency in specific miRNA target genes of *mir11707^{ge}/Tak-1* mutants at 28°C.

The increases are relative to their respective wild-type Tak-1 plants. Tak-1_mutant represents the *mir11707^{ge}/Tak-1* mutant lines M1-17, M2-1, and M2-2. White bars correspond to data at 22°C, and gray bars correspond to data at 28°C. Each line is shown as a white dot.

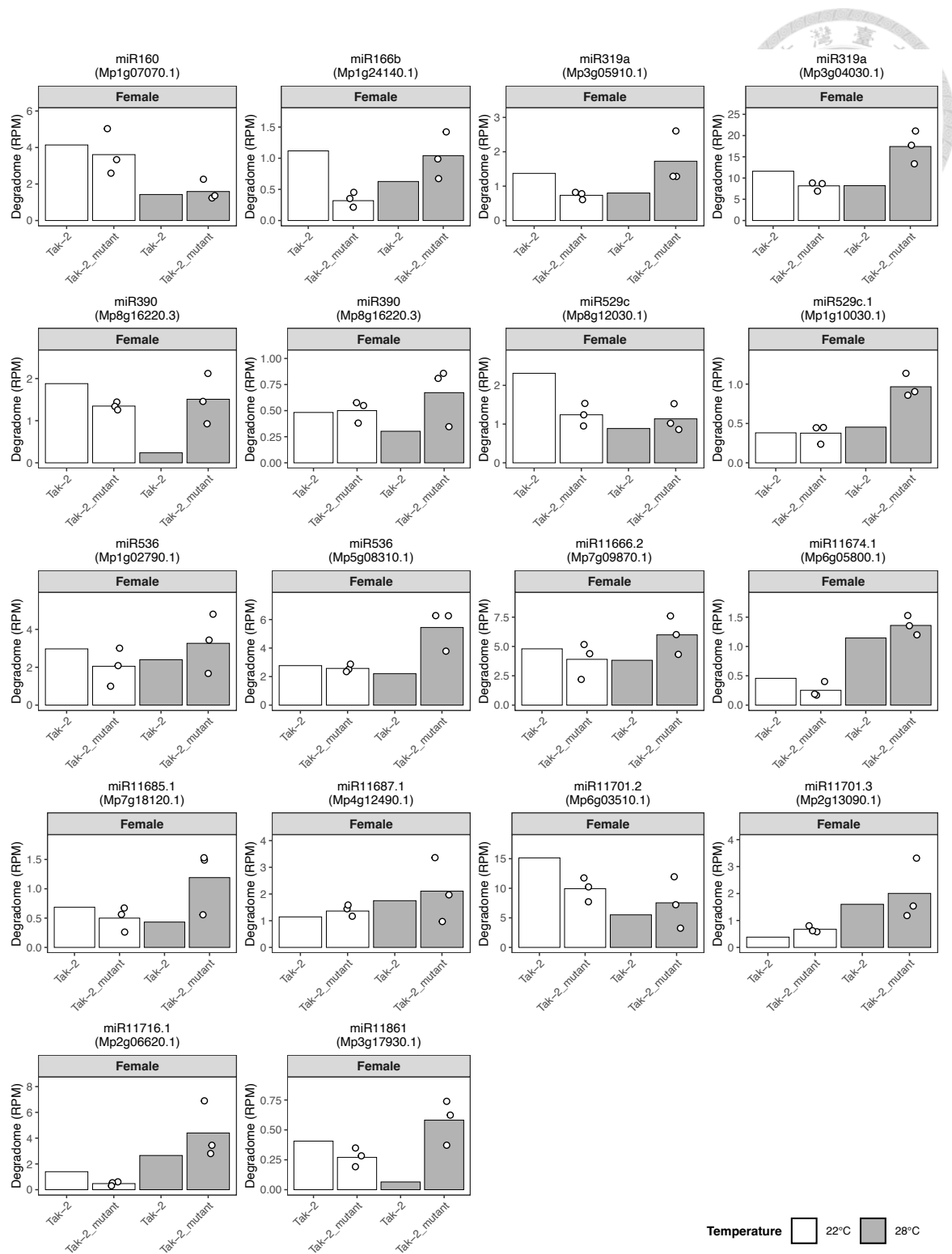


Figure 8. Increased RISC cleavage efficiency in specific miRNA target genes of

***mir11707^{ge}*/Tak-2 mutants at 28°C.**

The increases are relative to their respective wild-type Tak-2 plants. Tak-2_mutant represents the *mir11707^{ge}*/Tak-2 mutant lines F1-8, F2-1, and F2-3. White bars correspond to data at 22°C, and gray bars correspond to data at 28°C. Each line is shown as a white dot.



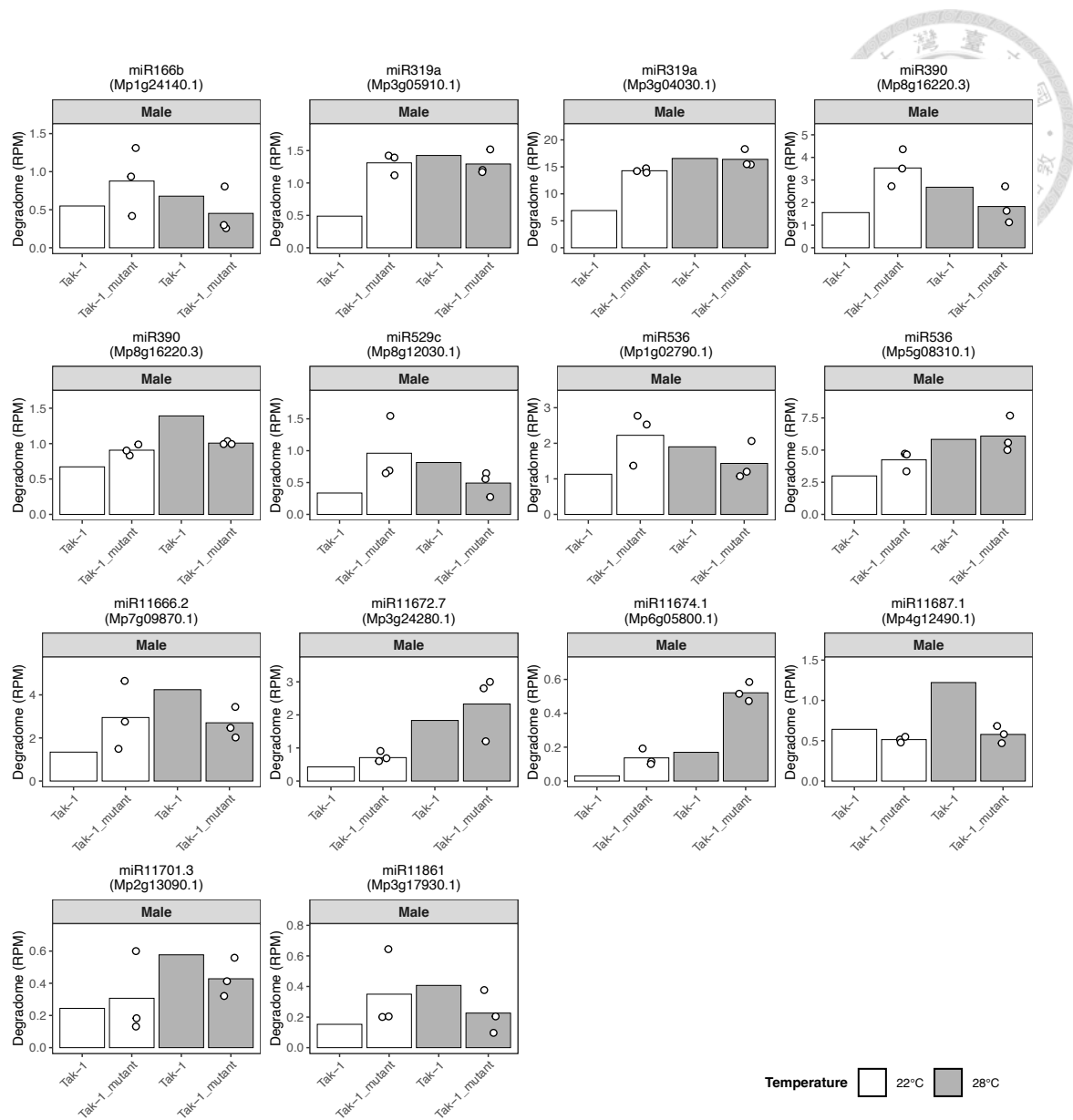


Figure 9. Increased RISC cleavage efficiency at 28°C compared to 22°C in Tak-1 plants.

White bars correspond to data at 22°C, and gray bars correspond to data at 28°C. Tak-1 mutant represents the *mir11707^{ge}*/Tak-1 mutant lines M1-17, M2-1, and M2-2. Each individual line is represented by a white dot.

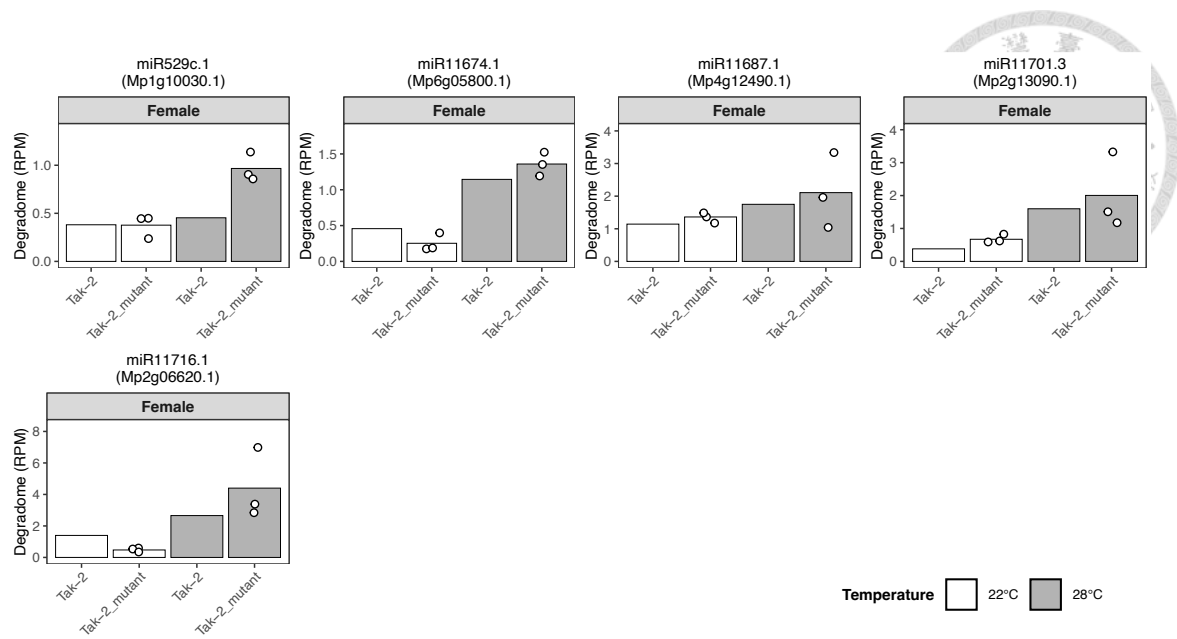


Figure 10. Increased RISC cleavage efficiency at 28°C compared to 22°C in Tak-2 plants.

White bars correspond to data at 22°C, and gray bars correspond to data at 28°C. Tak-2_mutant represents the *mir11707^{ge}*/Tak-2 mutant lines F1-8, F2-1, and F2-3. Each individual line is represented by a white dot.

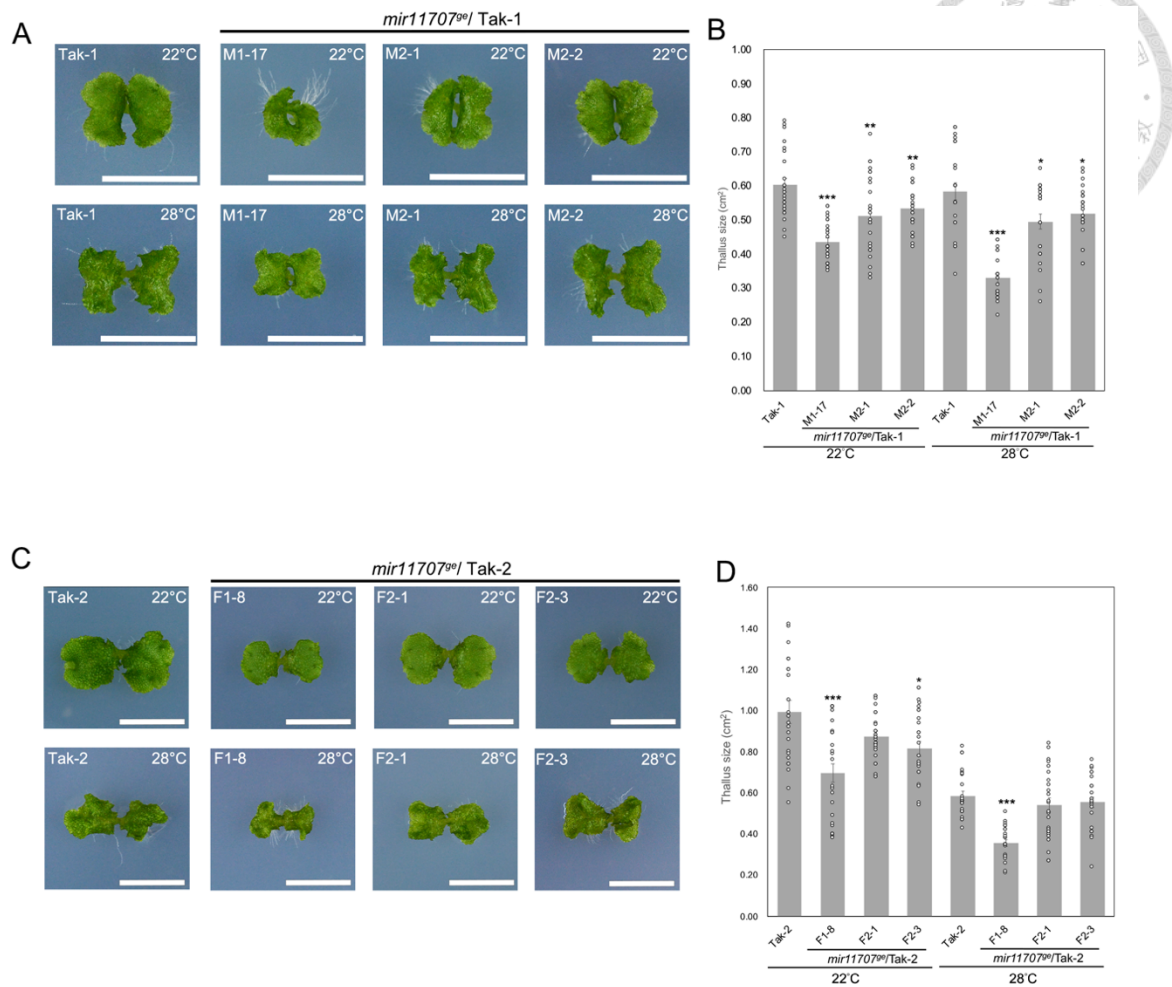
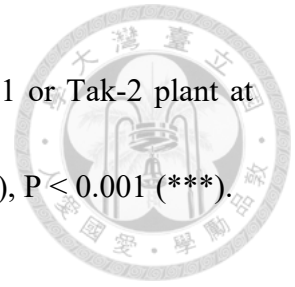


Figure 11. Phenotypic observations in 14-day-old *mir11707^{ge}* mutants at high temperature.

(A) *mir11707^{ge}/Tak-1* mutant lines and **(C)** *mir11707^{ge}/Tak-2* mutant lines 14-days-old, grown at 22°C and 28°C. Scale bar: 1 cm. **(B)** Top view and quantification of thallus area in *mir11707^{ge}/Tak-1* mutant lines. **(D)** Top view and quantification of thallus area in *mir11707^{ge}/Tak-2* mutant lines. Each data point, derived from a minimum of 25 independent plants, is represented by a white dot. Error bars indicate $\pm$ SE. P-values from

t-tests comparing each mutant line to its respective wild-type Tak-1 or Tak-2 plant at different temperatures are shown (n = 24); $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).



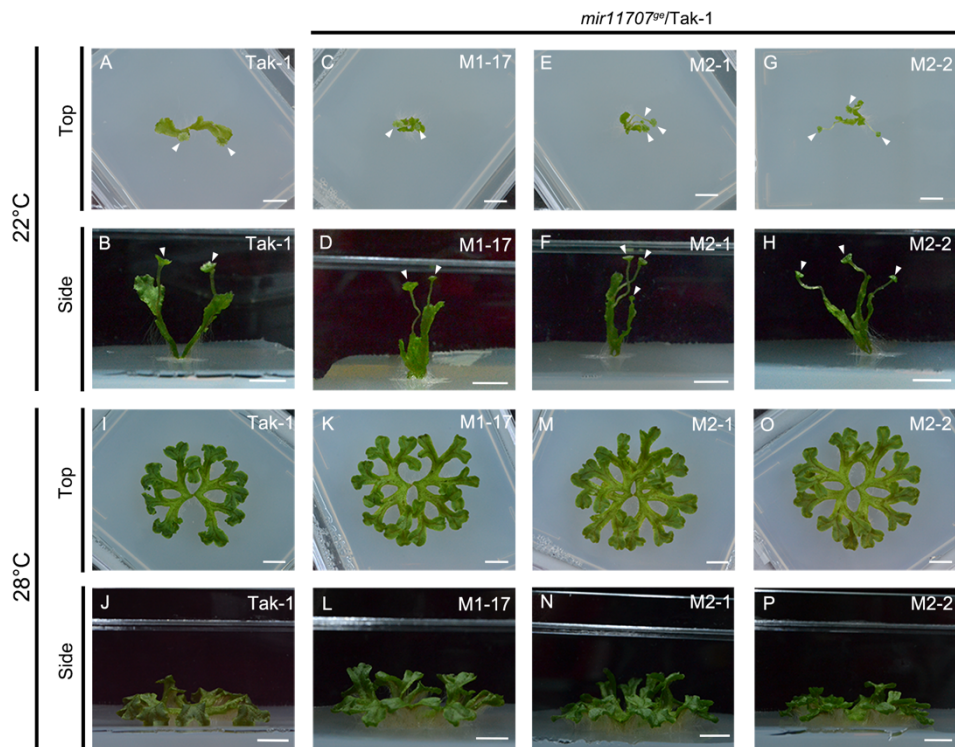


Figure 12. Phenotypic observation of sexual organs induced in 28-day-old *mir11707^{ge}/Tak-1* mutant lines under high temperature.

Tak-1 and *mir11707^{ge}/Tak-1* mutant lines grown at 22°C (**A-H**) and 28°C (**I-P**) induced sex organs under far-red light. For each temperature, the top panel shows the top view, and the bottom panel shows the side view. White arrows point to the sexual organs. Scale bar: 1 cm.

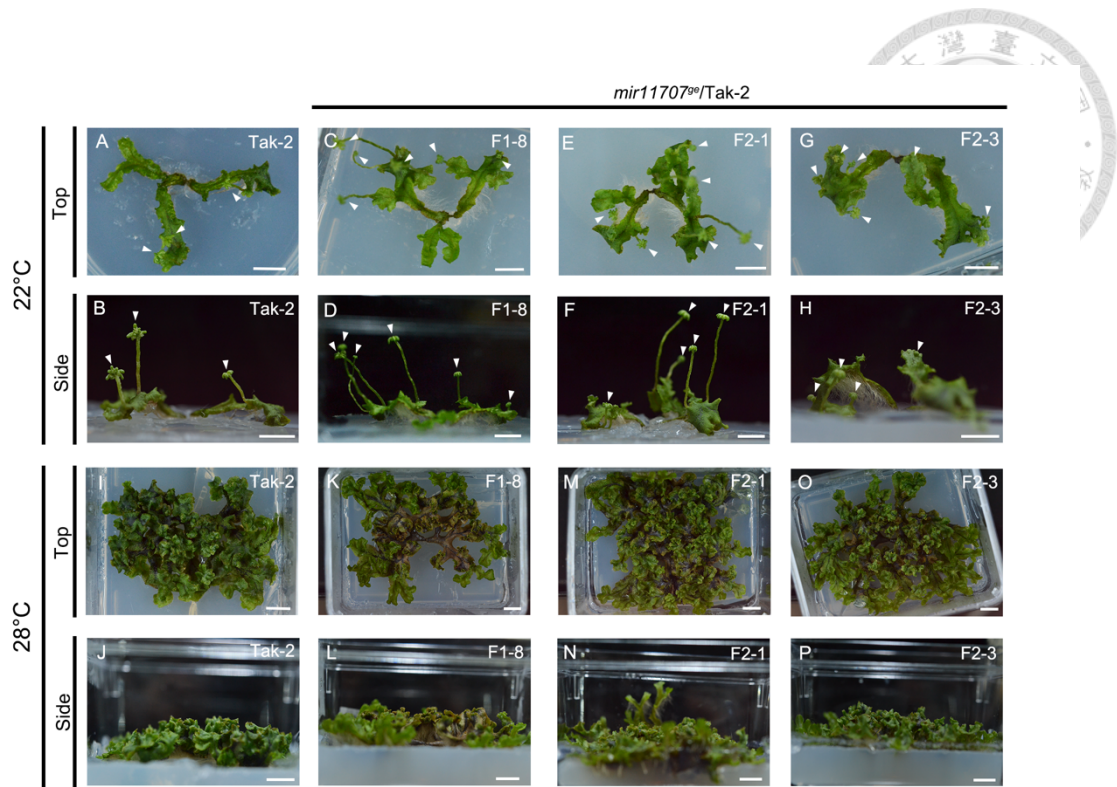


Figure 13. Phenotypic observation of sexual organs induced in 77-day-old *mir11707^{ge}/Tak-2* mutant lines under high temperature.

Tak-2 and *mir11707^{ge}/Tak-2* mutant lines grown at 22°C (A-H) and 28°C (I-P) induced sex organs under far-red. For each temperature, the top panel shows the top view, and the bottom panel shows the side view. White arrows point to the sexual organs. Scale bar: 1 cm.

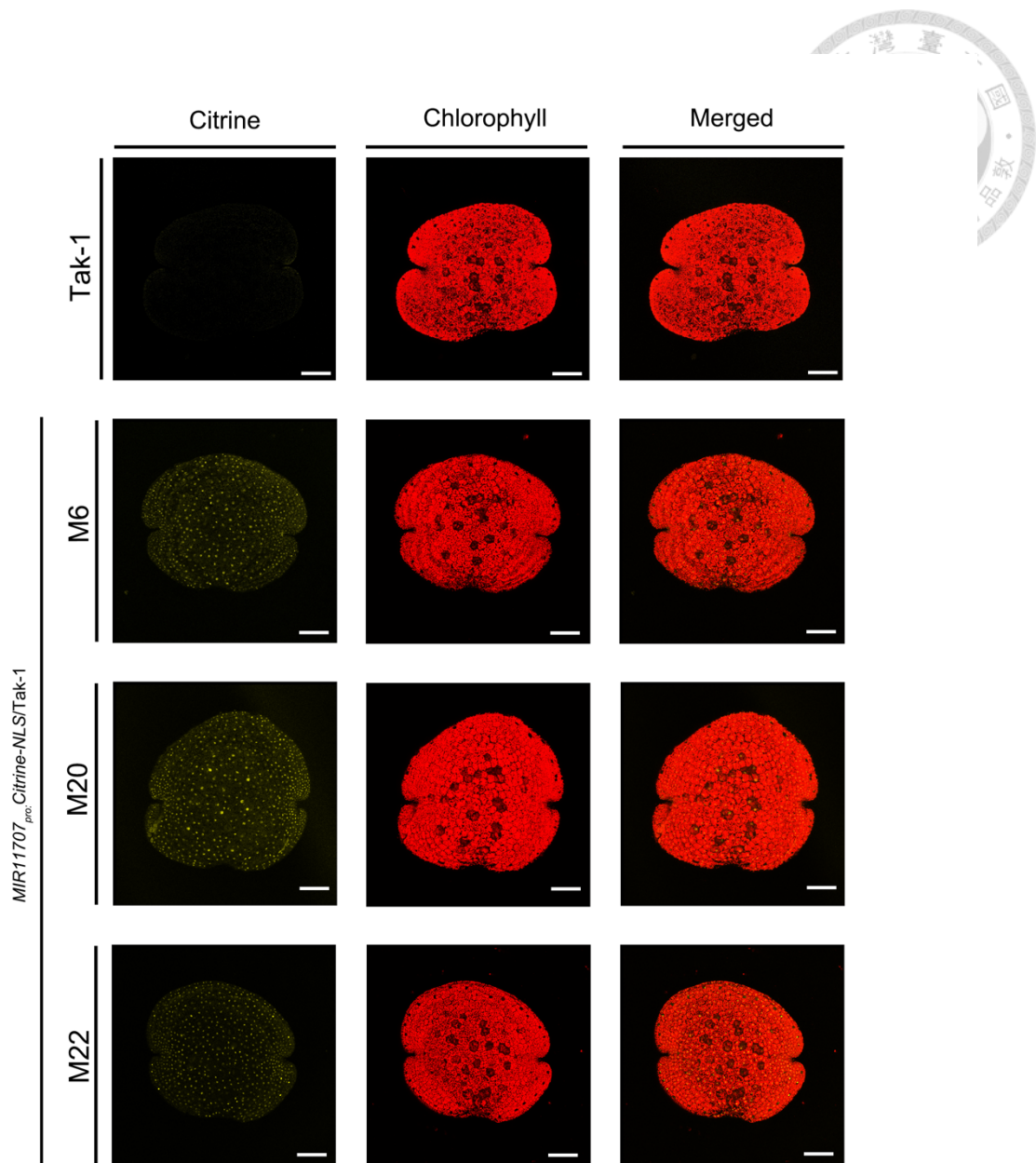


Figure 14. Expression patterns of the *MIR11707_{pro}:Citrine-NLS/Tak-1* in *M. polymorpha*.

Confocal images of the *MIR11707_{pro}:Citrine-NLS/Tak-1* plants, Tak-1 plant was used as a negative control. Scale bar: 100 μm .

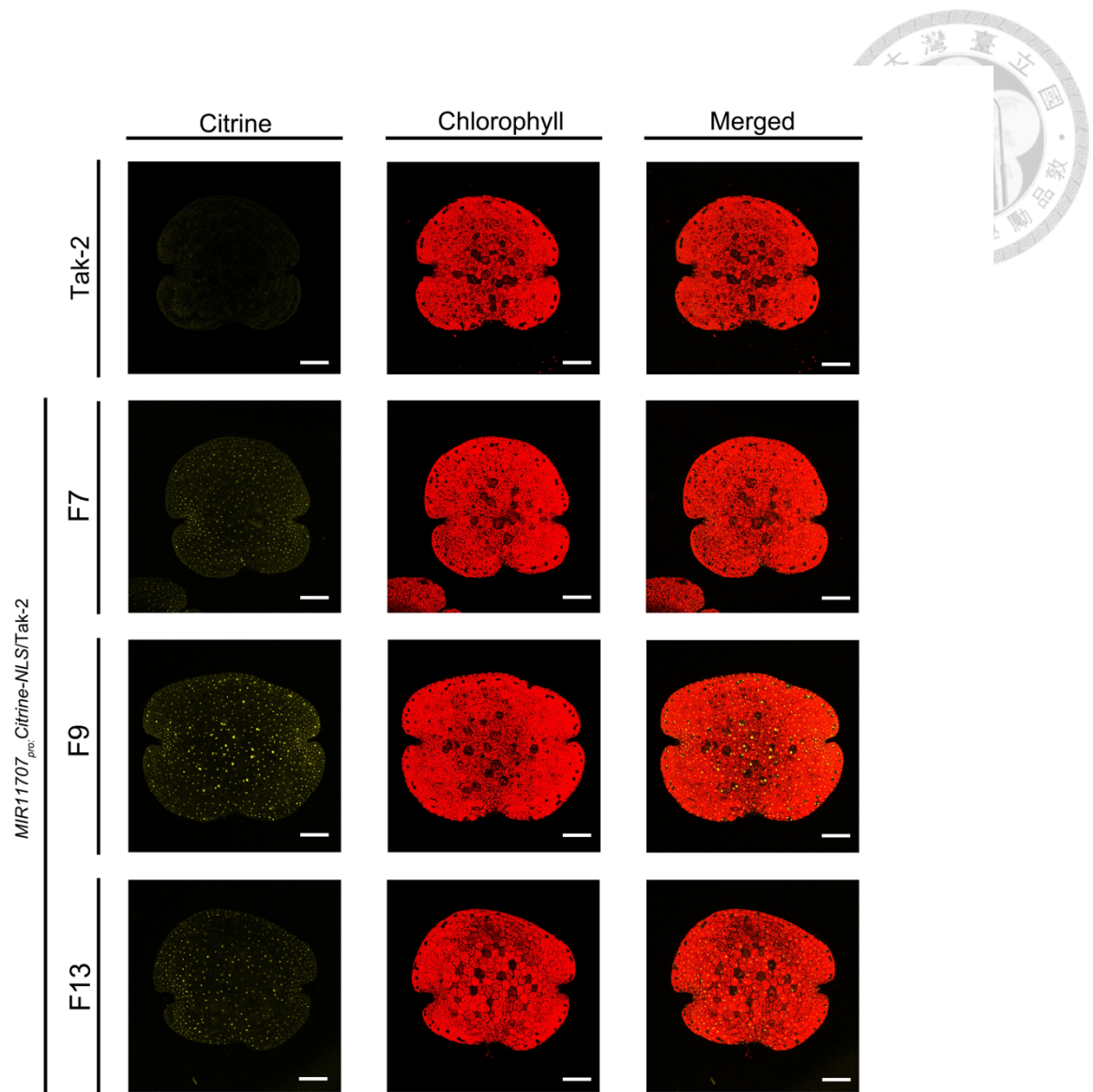


Figure 15. Expression patterns of the *MIR11707_{pro}:Citrine-NLS/Tak-2* in *M. polymorpha*.

Confocal images of the *MIR11707_{pro}:Citrine-NLS/Tak-2* plants, Tak-2 plant was used as a negative control. Scale bar: 100 μ m.

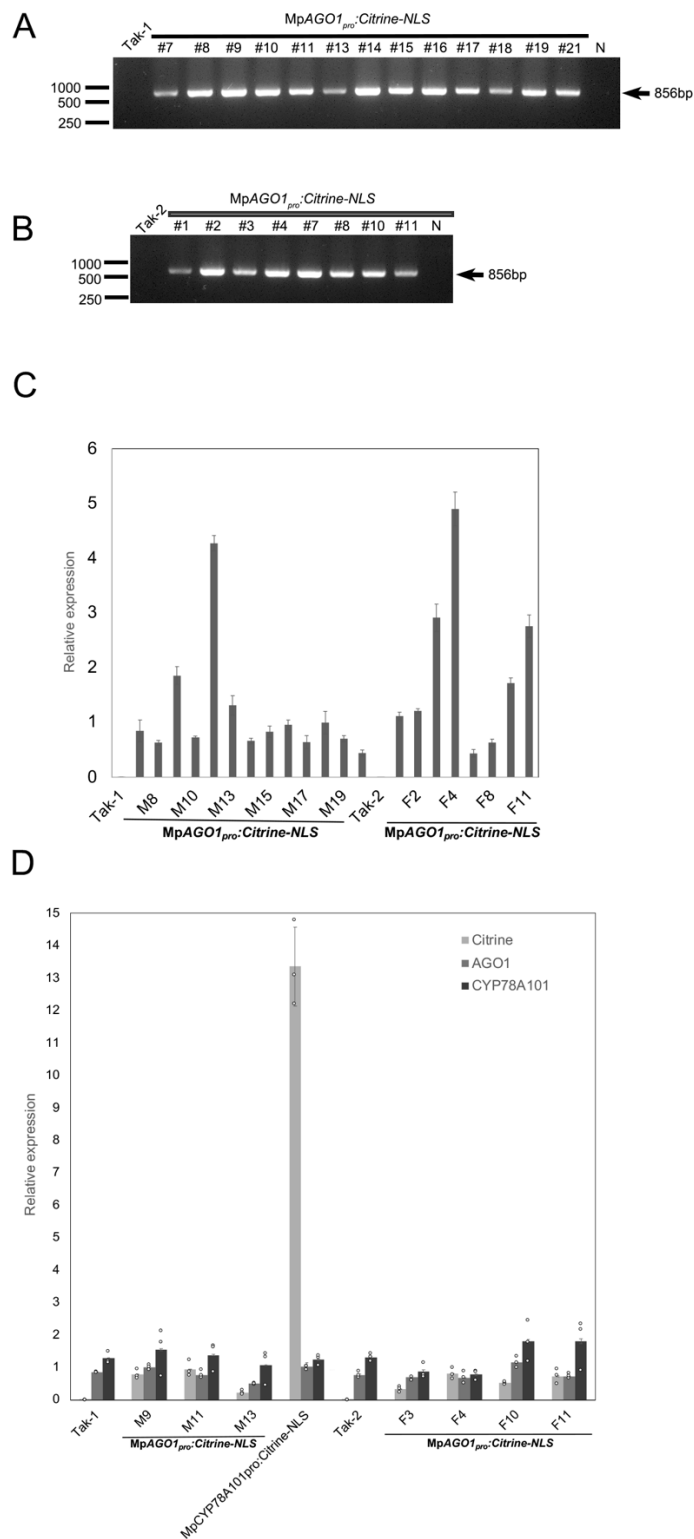
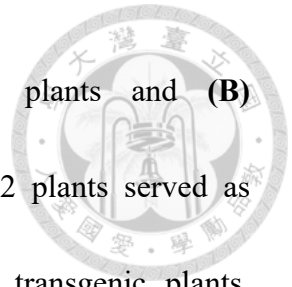


Figure 16. Promoter assay of *MpAGO1*_{pro}:Citrine-NLS in *M. polymorpha*.

Genotyping of (A) *MpAGO1_{pro}:Citrine-NLS*/Tak-1 transgenic plants and (B) *MpAGO1_{pro}:Citrine-NLS*/Tak-2 transgenic plants. Tak-1 and Tak-2 plants served as negative controls. (C) qRT-PCR of in *MpAGO1_{pro}:Citrine-NLS* transgenic plants. *MpEF1-alpha* was used as a reference gene. Tak-1 and Tak-2 plants served as negative controls. (D) qRT-PCR of *Citrine-NLS*, *MpAGO1*, *MpCYP78A101* in *MpAGO1_{pro}:Citrine-NLS* transgenic plants and *MpCYP78A101_{pro}:Citrine-NLS*/Tak-1 plants which were used as the experimental control. Tak-1 and Tak-2 plants served as negative controls. Error bars represent the standard error from three biological replicates, with *MpEF1-alpha* was used as a reference gene.



Supplementary Figure



Supplementary Figure 1. Degradome analysis of 34 miRNA target genes.

White bars correspond to data at 22°C, and gray bars correspond to data at 28°C. Tak-1_mutant represents the *mir11707^{ge}*/Tak-1 mutant lines M1-17, M2-1, and M2-2, while Tak-2_mutant represents the *mir11707^{ge}*/Tak-2 mutant lines F1-8, F2-1, and F2-3. Each individual line is represented by a white dot.