2018 年度　公益財団法人日本台湾交流協会　フェローシップ事業成果報告書
（自然科学分野）

探索 miR390-TAS3-ARF 在地錢中之原始功能

Exploring ancestral function of miR390-TAS3-ARF in Marchantia polymorpha

洪育翎
国立台湾大学植物科学所
招聘期間（2018 年 8 月 19 日～9 月 17 日）

2019 年
公益財団法人日本台湾交流協会

Contents

1. Background information

In plants, microRNAs (miRNAs) play significant roles for regulating development and growth, including developmental timing, cell proliferation, organ polarity, etc. (Cai et al., 2009; Rubio-Somoza and Weigel, 2011). Most of miRNA mediate gene silencing at posttranscriptional level by cleaving the complementary mRNA targets, thereby affecting developmental progress and growth in plants. The miRNA responsible for controlling the molecular mechanism of zygomorphic flowers was discovered by comparing small RNA seq. of dorsal and ventral petals in *S. speciosa*. Our lab found miR390 highly expressed in ventral petal and degraded its downstream gene *AUXIN RESPONSIVE FACTOR* (*ARF*) (validating by transcriptome, degradome, and qRT-PCR). By contrast, ARF highly expressed in dorsal petals and promoted growth of dorsal petals, the zygomorphic flower thus from (Nien, 2018). This regulatory pathway conserved in controlling the adaxial-abaxial polarity of leaf (Braybrook and Kuhlemeier, 2010; Chitwood et al., 2009) has been co-opted in floral zygomorphy (Nien, 2018).

MiR390 is a special type of miRNA, it cleaves a non-coding RNA, *TRANS-ACTING SIRNA 3* (*TAS3*), generating 21-nucleotide small RNAs, *trans*-acting small interfering RNAs (tasi-RNAs). Then, tasi-RNAs (ta-siARF) specifically degrade the targeted *ARF* genes, which is crucial for auxin signals. This molecular mechanism calls miR390-*TAS3*-*ARF* pathway. In addition, miR390 is a conserved miRNA, the regulatory target of miR390 is also conserved in land plant (Axtell and Bowman, 2008). Many conserved miRNAs show abundantly expressed, and their functions are to regulate the transcript factors involved in development control (Axtell and Bowman, 2008). Indeed, in angiosperm, this conserved miR390-*TAS3*-*ARF* pathway plays an important role in developmental progression (Rubio-Somoza and Weigel, 2011), reproductive development (Ding et al., 2018), root architecture (Marin et al., 2010), and adaxial-abaxial polarity of leaf and flower (Braybrook and Kuhlemeier, 2010; Chitwood et al.,

2009; Nien, 2018). Actually, this pathway is not unique in angiosperm, it emerges from the most ancient extant lineage of land plants, *Marchantia polymorpha* (liverwort) (Xia et al., 2017). However, the ancestral role of miR390-*TAS3-ARF* pathway, which is firstly emerging from *M. polymorpha*, is remaining unclear. Even the understanding of the role of miR390-*TAS3-ARF* pathway in fern (monilophytes) and other basal land plants (bryophyte) are limited. In *Physcomitrella* patens (moss), belonging to bryophyte, miR390 cooperates with miR156 to control the phase transition (from young protonemata to adult leafy gametophores) (Cho et al., 2012). This study revealed that the possibility of the ancestral role of miR390-*TAS3-ARF* pathway, which is important in phase transition. However, the target genes by ta-siRNAs are different between *M. polymorpha* and *P. patens*. In bryophyte, mi390 cleaves TAS3 to generate not only ta-siARF but also ta-siAP2, which degrade *APETALA2* (*AP2*). In *P. patens*, both of ta-siARF and ta-siAP2 degrade to its target gene and contribute to phase transition (Cho et al., 2012). By contrast, in *M. polymorpha*, no target gene of ta-siAP2 was found and degraded (Xia et al., 2017). This implies that the regulator mechanism of miR390-*TAS3-ARF* pathway might be a little different between *P. patens* and *M. polymorpha*. To explore the ancestral role of miR390-*TAS3-ARF* pathway, *M. polymorpha* should be a better material.

M. polymorpha represents the most ancient extant lineage of land plants, which exhibits low genetic redundancy in most regulatory pathways (Bowman et al., 2017). Indeed, all the components of miR390-*TAS3-ARF* pathway show only one copy in *M. polymorpha* (Xia et al., 2017). In addition, The nuclear and organellar genomes of *M. polymorpha* has been sequenced (Bowman et al., 2016), the sequence, structure, annotation, promoter sequence, the copy number of genes, miRNAs information (Lin et al., 2016) can be available on the website: Marpolbase (http://marchantia.info/). Moreover, *Agrobacterium*-mediated transformation system and CRISPR/Cas9

knockout system have been well developed in *M. polymorpha* (Ishizaki et al., 2008; Shen et al., 2014; Tsuboyama-Tanaka and Kodama, 2015), the transformants could be available within one month. A series of Gateway binary vectors designed for transgenic experiments on *M. polymorpha* are also well development (Ishizaki et al., 2015). All of these advantages promote the progress of research in *M. polymorpha*.

To validate the potential function of miR390-*TAS3-ARF* pathway, I will firstly examine the expression pattern of all the members of this pathway by qRT-PCR in different developmental stages and different organs in *M. polymorpha*. Also, reporter assay was applied to check the precisely expression site of this pathway. After knowing the expression pattern of miR390-*TAS3-ARF* pathway, I shall be able to validate the potential development role in this pathway if they have specifically expressed in certain developmental stages or organs in *M. polymorpha*. To further explore the phenotypic effects of miR390-*TAS3-ARF* pathway in *M. polymorpha*, I firstly focus on the upstream gene, miR390. I will use genetic transformation approach by overexpressing and knocking out miR390 to observe the phenotypic effects in *M. polymorpha*. Although miR390-*TAS3-ARF* pathway is conserved in the regulation of dorsi-ventral asymmetry in both of leaf (Braybrook and Kuhlemeier, 2010; Chitwood et al., 2009) and flower (Nien, 2018) in flowering plants. *M. polymorpha* didn't show the differently expression in thallus (leaf-like structure) (Ryuichi Nishihama, personal communication 2018). Therefore, we assume that the role of miR390-*TAS3-ARF* pathway in *M. polymorpha* might be the similar to that in *P. patens* due to the closely phylogenetic relationship. In addition, it has reported that phase transition is regulated by miR156, miR172, and miR390 in flowering plants (Fahlgren et al., 2006; Wu et al., 2009). Interestingly, among aforementioned three miRNAs, only miR390 exists in *M. polymorpha* (Tsuzuki et al., 2016). It will be worth investigating whether the miR390 controls the phase transition instead of miR156 and miR172 in *M. polymorpha*. While

we focus on phase transition in *M. polymorpha*, we also carefully observe the phenotypic effects of miR390 to understand whether it has any roles in gametophyte and/or gametangia development.

2. Specific aims

Aim 1: To explore the potential function of miR390-*TAS3-ARF* pathway, we will detect the expression pattern (via real time RT-PCR and reporter assay) of all the members of this pathway in *M. polymorpha*. If miR390 and ARF specifically expressed in certain tissue/organ or developmental stages, we could infer their potential developmental roles in liverwort.

Aim 2: To further investigate the phenotypic effects of miR390-*TAS3-ARF* pathway genes, we will overexpress and knock out miR390 and ARF in *M. polymorpha*.

3. Significance

For functional study of miR390-*TAS3-ARF* pathway, we can demonstrate the role of this pathway in developmental control in *M. polymorpha* and extend our understanding on the evolution of miR390 from bryophytes to angiosperm.

4. Preliminary data and Research design

4.1. Investigate the expression pattern of miR390-*TAS3-ARF* pathway in *M. polymorpha* (Research design)

4.1.1. validate temporal and spatial regulation of miR390 and its targets by qRT-PCR

In order to check the role of miR390-*TAS3-ARF* pathway in the development of *M. polymorpha*, we will detect the expression pattern of miR390 and its target in different developmental stages and different organs after spores germinating.

Method:

(1) we will collect 1 to 8-week germinated spores which are cultured on half strength Gamborg's B5 medium for detecting the expression level of miR390, *TAS3*, ta-siARF, and *ARF2*.

(2) We will choose different organs, such as thalli, gemmae, gametangium, and sporophyte, to detect the expression level of miR390, *TAS3*, ta-siARF, and *ARF2* at the miR390 highly expressed stage.

Detail: We will use stem-loop strategy for confirming the expression level of microRNA and small RNA, miR390 and ta-siARF. The stem-loop RT primer specific to mature miR390 and ta-siARF were used for reverse transcription, then, the cDNA products were used for qRT-PCR. And U6 was used as the endogenous control. For *TAS3* and *ARF2*, we will detect the expression level by qRT-PCR.

Expected results:

(1) Due to both of *M. polymorpha* and *P. patens* belonging to bryophyte, we assume the function of miR390 might be similar. We expect that miR390 also regulate the phase transition in *M. polymorpha*, therefore, the expression of miR390 might be high and/or continued before phase transition. The expression of ta-siARF should be consistent with miR390. By contrast, the expression of *ARF2* will be low because of the degradation by ta-siARF.

4.1.2. check temporal and spatial regulation of miR390 and its targets by reporter

Method:

(1) Using the material described in 7.3.1 to check the expression site of miR390 and *TAS3* express by reporter assay (The ARF2 has been detected in Kyoto University). The sequence of promoter fragment could be downloaded from MarpolBase. In addition, the length of promoter is suggested to around 5 kb because the distance

between each gene is large (>10 kb) in *M. polymorpha* (Kyoto University, personal communication 2018). Both of GUS and Citrine will be used as reporter gene to detect the precisely expression site of miR390 and TAS3.

Detail: Promoter sequence of miR390 (Mapoly0062s0002) and TAS3 (Mapoly0154s0042) were obtained from MarpolBase. *pMIR390:GUS/Citrine* and *pTAS3:GUS/Citrine* report vectors will be built by amplifying the promoter region for 4.5 and 5.6 kb, respectively. The amplified pMIR390 and pTAS3 were ligate into entry vector pENTR1A and recombined into pMpGWB304 (GUS reporter) and pMpGWB315 (Citrine reporter) vector (pMpGWB304 and pMpGWB315 were gifts from Takayuki Kohchi). Marchantia sporelings were transformed by *Agrobacterium* strain GV2260 harboring binary vectors with reporter gene. Next, the transformants are selected by antibiotic (0.5 μM chlorosulfuron).

Expected results:

(1) The expression pattern might correspond to the results of 7.3.1, but we can precisely understand the expression site of miR390 and TAS3.

4.2. **Validate the function of miR390 in *M. polymorpha* (Research design)**

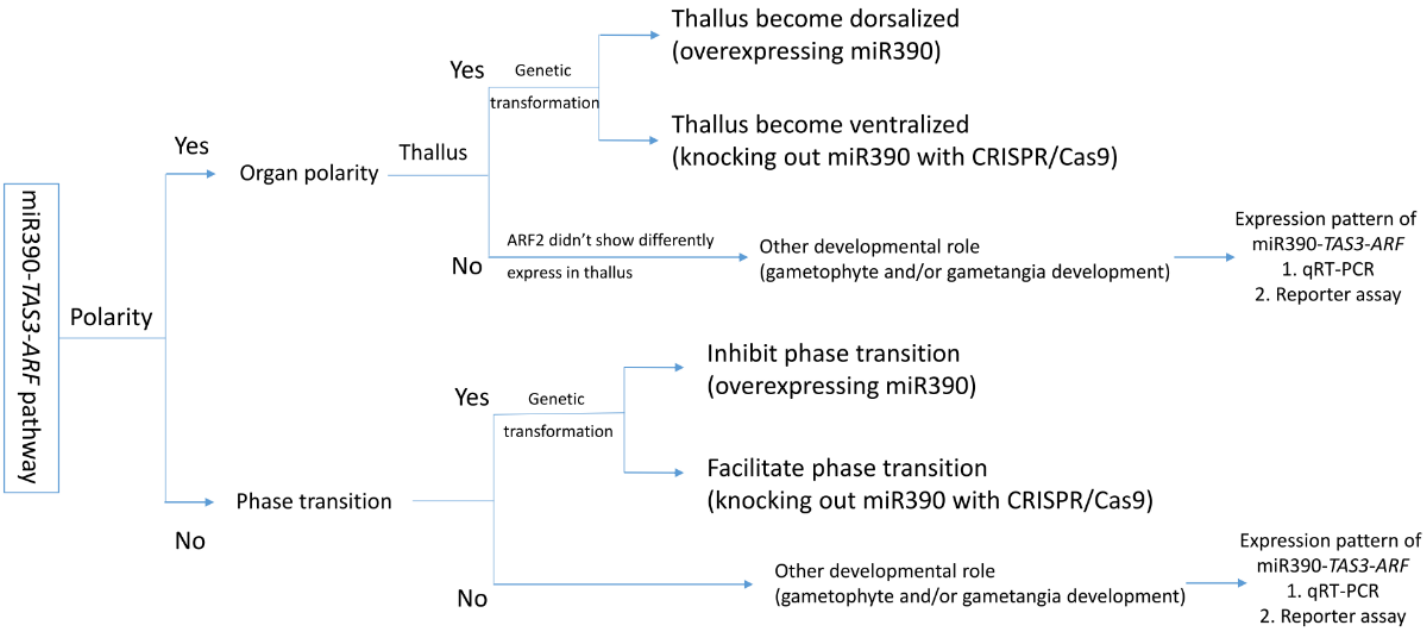
Method:

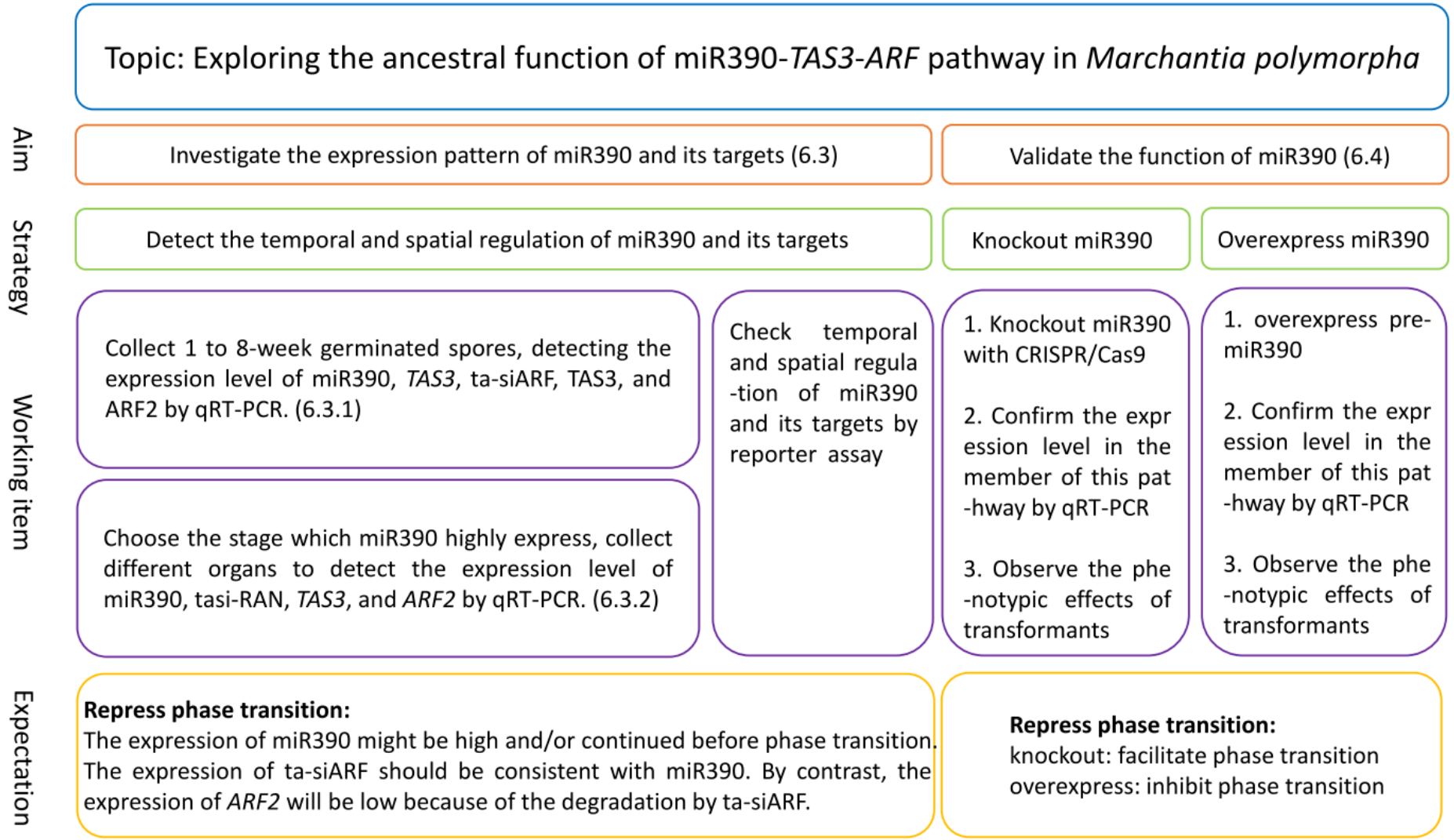
(1) To confirm the function of miR390 in *M. polymorpha*, *Agrobacterium* overexpressing and knocking out miR390 with CRISPR/Cas9 will be conducted. After getting the transformants, the expression level of miR390, ta-siARF, and ARF2 will be checked for confirming the successfully transformation by qRT-PCR. Moreover, the direct sequencing of mutant individuals will be conducted to check the editing event of miR390 by CRISPR/Cas9. Then, the phenotype of transformants will be carefully observed whether miR390 has any role in (1) phase transition and (2) gametophyte and/or gametangia development.

Detail: For knocking out miR390, the miR390 sequence in *M. polymorpha* was obtained from MarpolBase. The guide RNA (gRNA) of miR390 was designed on the website, CRISPRdirect. Then, the gRNA was ligated into entry vector pMpGE_En03 with MpU6 promoter and LR recombined into binary destination vector pMpGE010 (pMpGE_En03 and pMpGE010 were gifts from Takayuki Kohchi). For overexpressing miR390, the vector for miR390 overexpression was constructed by insertion of pre-miR390 into pMpGWB303 with MpEF1alpha promoter, which shows strong activity than 35S promoter in *M. polymorpha* (pMpGWB303 was a gift from Takayuki Kohchi, Addgene plasmid # 68631). *Agrobacterium*-mediated transformation will be conducted to get the transformants.

Expected results:

(1) We already knew that miR390 repressed phase transition in *P. patens*. Therefore, we expect that the transgenic plants with overexpressed miR390 might delay the phase transition. In contrast, the knockout miR390 plant might promote phase transition in *M. polymorpha*.




Topic: Exploring the ancestral function of miR390-*TAS3-ARF* pathway in *Marchantia polymorpha*
Aim
Investigate the expression pattern of miR390 and its targets (6.3)
Validate the function of miR390 (6.4)
Strategy
Detect the temporal and spatial regulation of miR390 and its targets
Knockout miR390
Overexpress miR390
Working item
Collect 1 to 8-week germinated spores, detecting the expression level of miR390, *TAS3*, ta-siARF, TAS3, and ARF2 by qRT-PCR. (6.3.1)
Choose the stage which miR390 highly express, collect different organs to detect the expression level of miR390, tasi-RAN, *TAS3*, and *ARF2* by qRT-PCR. (6.3.2)
Check temporal and spatial regula -tion of miR390 and its targets by reporter assay
1. Knockout miR390 with CRISPR/Cas9
2. Confirm the expr ession level in the member of this pat -hway by qRT-PCR
3. Observe the phe -notypic effects of transformants
1. overexpress pre-miR390
2. Confirm the expr ession level in the member of this pat -hway by qRT-PCR
3. Observe the phe -notypic effects of transformants
Expectation
Repress phase transition:
The expression of miR390 might be high and/or continued before phase transition. The expression of ta-siARF should be consistent with miR390. By contrast, the expression of *ARF2* will be low because of the degradation by ta-siARF.
Repress phase transition:
knockout: facilitate phase transition
overexpress: inhibit phase transition

References

Axtell, M.J., and Bowman, J.L. (2008). Evolution of plant microRNAs and their targets. Trends Plant Sci *13*, 343-349.

Bowman, J.L., Araki, T., Arteaga-Vazquez, M.A., Berger, F., Dolan, L., Haseloff, J., Ishizaki, K., Kyozuka, J., Lin, S.S., Nagasaki, H.*, et al.* (2016). The Naming of Names: Guidelines for Gene Nomenclature in Marchantia. Plant Cell Physiol *57*, 257-261.

Bowman, J.L., Kohchi, T., Yamato, K.T., Jenkins, J., Shu, S., Ishizaki, K., Yamaoka, S., Nishihama, R., Nakamura, Y., Berger, F.*, et al.* (2017). Insights into Land Plant Evolution Garnered from the Marchantia polymorpha Genome. Cell *171*, 287-304 e215.

Braybrook, S.A., and Kuhlemeier, C. (2010). How a Plant Builds Leaves. The Plant Cell *22*, 1006-1018.

Cai, Y., Yu, X., Hu, S., and Yu, J. (2009). A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics *7*, 147-154.

Chitwood, D.H., Nogueira, F.T., Howell, M.D., Montgomery, T.A., Carrington, J.C., and Timmermans, M.C. (2009). Pattern formation via small RNA mobility. Genes Dev *23*, 549-554.

Cho, S.H., Coruh, C., and Axtell, M.J. (2012). miR156 and miR390 Regulate tasiRNA Accumulation and Developmental Timing in Physcomitrella patens. The Plant Cell *24*, 4837-4849.

Ding, B., Xia, R., Gurung, V., Sagawa, J.M., Stanley, L.E., Strobel, M., Lin, Q., Diggle, P.K., Meyer, B.C., and Yuan, Y.-W. (2018). Developmental genetics of corolla tube formation: role of the tasiRNA-ARF pathway. bioRxiv.

Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., and Carrington, J.C. (2006). Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. Curr Biol *16*, 939-944.

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., 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.

Lin, P.-C., Lu, C.-W., Shen, B.-N., Lee, G.-Z., Bowman, J.L., Arteaga-Vazquez, M.A., Liu, L.-Y.D., Hong, S.-F., Lo, C.-F., Su, G.-M.*, et al.* (2016). Identification of miRNAs and Their Targets in the Liverwort Marchantia polymorpha by Integrating RNA-Seq and Degradome Analyses. Plant and Cell Physiology *57*, 339-358.

Marin, E., Jouannet, V., Herz, A., Lokerse, A.S., Weijers, D., Vaucheret, H., Nussaume, L., Crespi, M.D., and Maizel, A. (2010). miR390, Arabidopsis TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. Plant Cell *22*, 1104-1117.

Nien, Y.C. (2018). Global analysis of small RNAs for controlling floral symmetry in Sinningia speciosa. Master thesis National Taiwan University.

Rubio-Somoza, I., and Weigel, D. (2011). MicroRNA networks and developmental plasticity in plants. Trends Plant Sci *16*, 258-264.

Santos-Rosa, M., Poutaraud, A., Merdinoglu, D., and Mestre, P. (2008). Development of a transient expression system in grapevine via agro-infiltration. Plant Cell Rep *27*, 1053-1063.

Shen, B., Zhang, W., Zhang, J., Zhou, J., Wang, J., Chen, L., Wang, L., Hodgkins, A., Iyer, V., Huang, X.*, et al.* (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods *11*, 399-402.

Tsuboyama-Tanaka, S., and Kodama, Y. (2015). AgarTrap-mediated genetic transformation using intact gemmae/gemmalings of the liverwort Marchantia polymorpha L. J Plant Res *128*, 337-344.

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.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., and Poethig, R.S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell *138*, 750-759.

Xia, R., Xu, J., and Meyers, B.C. (2017). The Emergence, Evolution, and Diversification of the miR390-TAS3-ARF Pathway in Land Plants. Plant Cell *29*, 1232-1247.