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參展科別　植物學

作品名稱　**miRNAs 在光形態發生中的影響**

得獎獎項　大會獎：三等獎

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關鍵字　**miRNA、light、photomorphogenesis**

作者簡介

第一作者：

我是劉博鈞，目前就讀於建國中學科學班三年級，已知用火。對事物發生的原因，總是充滿好奇。在高一時，有幸進入素幸老師的實驗室，進行植物學相關的專題。從中學到許多的經驗及精神，對於相關的科學知識也增進不少，真的是一段美好的旅程。同時在科學之外，也喜歡閱讀及分享自己的所見所聞所思，而進行專題的經驗，也讓我在人生的閱歷上留下了精彩的一頁。而這每一段的驚喜，都讓自己的生活更為豐富。

第二作者：

我是建國中學數理資優班，陳霽佑同學。熱衷於吸收各類知識，因此愛好閱讀。隨著年紀的增長，對於科學的了解更為深入，也希望能進一步的學習。而在高一下，幸運地來到素幸老師的實驗室進行學習，學習做科學的精神，以及植物學研究的方法。學長、姐們對於科學研究的嚴謹與投入，不禁讓我對專題研究充滿熱情，透過這個專題，讓我了解到實驗研究的艱辛與不易，但也使我在過程中獲得思辨力與成就感。

摘要

在種子萌發的階段，光會造成幼苗的形態變化，稱為光形態發生(photomorphogenesis)。然而 microRNA (miRNA) 如何影響光形態發生，一直未被詳細探討。藉由次世代定序 (Next Generation Sequencing)之結果分析，得知阿拉伯芥（*Arabidopsis thaliana*）幼苗中，具有受光調控的 miRNA。利用北方墨點法 (northern blotting) 及即時聚合酶鏈鎖反應 (Real-Time PCR)，我們驗證了 miRNA 和其降解目標之相關性。在篩選過後選得 miR396s 和 miR858s，作為後續研究的目標。下一步便是了解他們的功能。目前已經證明 miRNA 及對應目標基因的突變株確實會影響花青素、葉綠素累積，下胚軸延長及子葉發育。考量到 miRNA 有功能性的替代物 (functional redundancy)，我們於是進行標的序列模擬(target mimicry)，以削減 miRNA 之功能；也在建立大量表現上述 miRNA 之轉殖株，了解其與光形態發生之關聯。最終目標為解開 miR396 與 miR858 對光形態發生的調控機制。

Abstract

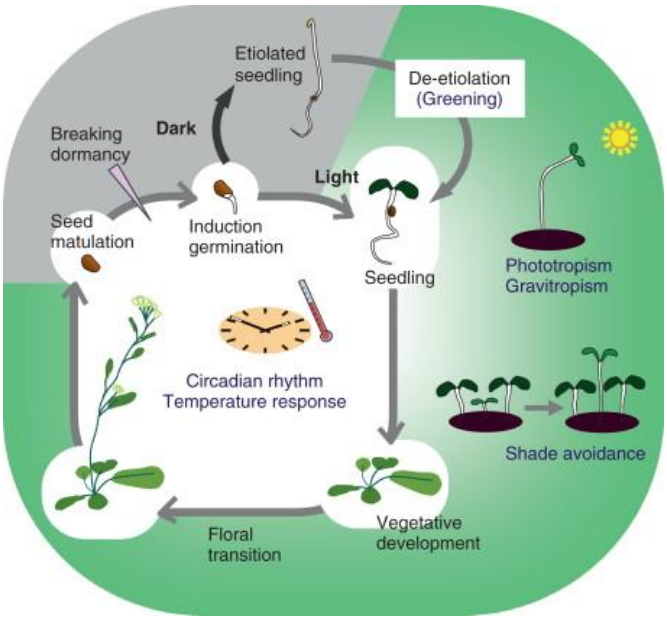
In order to respond to light, the seedlings have physical changes called photomorphogenesis. We hypothesize that miRNAs could be important regulators in photomorphogenesis. To test our hypothesis, we first conduct northern blotting and Real-Time PCR to verify the expressions of selected miRNA-target pairs from previous Next Generation Sequencing results. We finally choose miR396s and miR858s because the miRNA expression patterns in northern blotting results are light regulated. Through Real-Time PCR, we have confirmed the expression of the targets and validated anti-correlation between the miRNAs and selected targets. The next step is to study if the miRNA target mutants indeed show defectives in photomorphogenesis. We have screened for homozygous T-DNA insertion mutants of miRNAs / targets, which were used for photomorphogenic phenotype observation. So far, we have proven that the seedlings with mutated targets indeed have different anthocyanin and chlorophyll accumulation, cotyledons and hypocotyls. Considering that miR858 mutants cannot be acquired, and that there is functional redundancy inside the miR396 and miR858 families, to completely reduce the function of the selected miRNAs, we have introduced the target mimicry constructs of miR396 and miR858 into *Arabidopsis* Col-0 wild type. Also, we are screening for the overexpression lines of *MIR396B* and *MIR858A*. The plants will be used for photomorphogenic phenotypes observation. By screening and studying the photomorphogenic phenotypes, we can discover the function of miRNA-target pairs required for regulation of photomorphogenesis.

Introduction

A. Literature Review

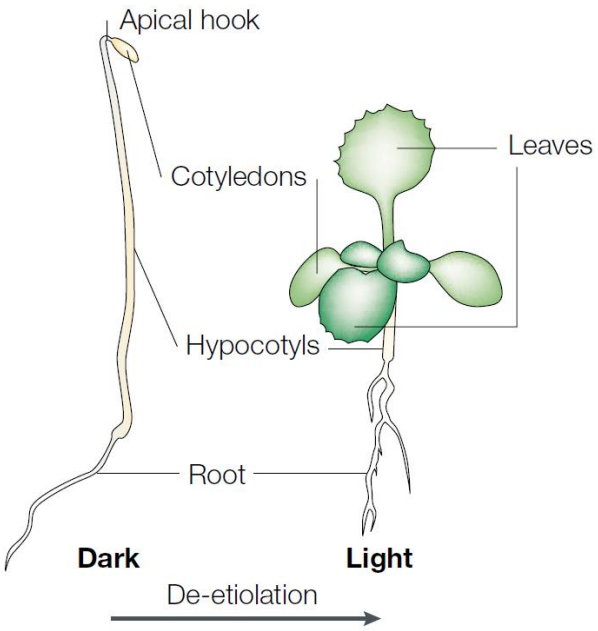
1. Photomorphogenesis

Light plays important roles in plant life. From seed germination to vegetative and reproductive growth, light has become one of the most important determinants of plant development (Figure 1). Photomorphogenesis is a survival strategy when the seedlings encounter light (Figure 2). In *Arabidopsis thaliana*, *HY5* is a transcription factor that plays important roles in transcription of light-responsive genes (Deshaies and Meyerowitz, 2000); hence, resulting in photomorphogenesis (Figure 3). As seeds under the ground, their hypocotyls will elongate in order to find light, and the cotyledons will become hook shaped so as to protect the shoot apical meristem. After emerging from soil surface, the cotyledons will develop when the plants respond to the light because they need to increase photosynthetic efficiency, and the hypocotyls will be de-etiolated under light (Bartel B & Bartel DP, 2003). Furthermore, anthocyanins will also accumulate because it is a good reductant that reduce free radical to prevent the plants from photodamage. Similarily, chlorophyll content will increase contributing to the higher efficiency of photosynthesis.



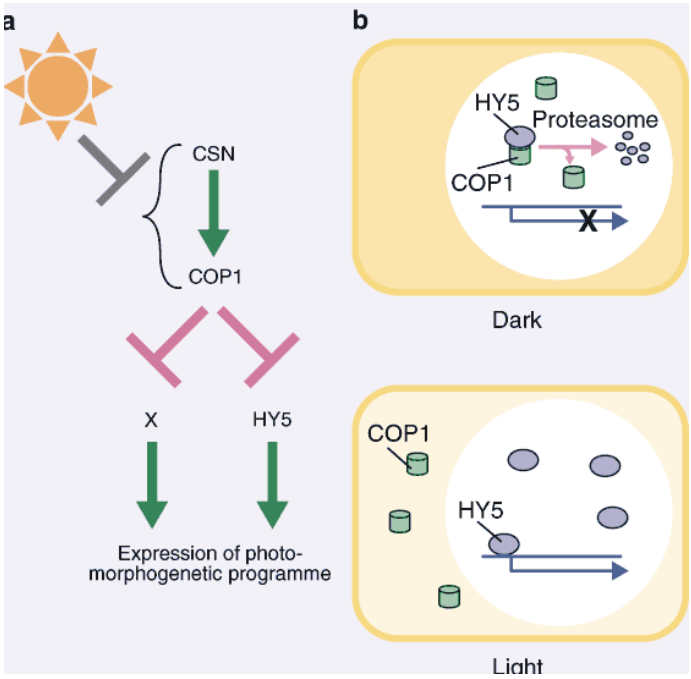
(Kami *et al.*, 2010)

Figure 1. The morphological changes induced by light and temperature over the lifetime of *Arabidopsis thaliana***.** In our project, we focus on de-etiolation, also called photomorphogenesis. The importance of this process is for the survival of seedlings upon light exposure.



(Quail, 2002)

Figure 2. Detailed morphological changes in *Arabidopsis* during photomorphogenesis, such as inhibited elongation of hypocotyls, opening of cotyledon, accumulation of anthocyanins and chlorophylls.



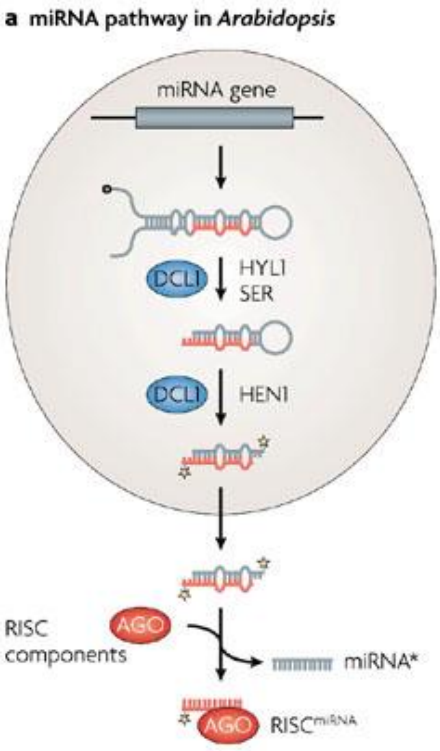
(Deshaies and Meyerowitz, 2000)

Figure 3. HY5 is a transcription factor acts as the main switch of photomorphogenesis. In dark, COP1 degrade HY5, so seedlings remain at etiolated state. When exposed to light, COP1 will move outside from nucleus and is unable to degrade HY5.

2. miRNA

miRNAs are small RNA sequences (21-24nt) which are involved in many biotic reactions in eukaryotes. They will bind to targets mRNAs with complementary sequences and the mRNA will be degraded by miRNA loaded RISC (RNA-induced silencing complex) (Jones-Rhoades & Bartel 2004, Jones-rhoades et al., 2006). RISC is known to cause post-transcriptional mRNA cleavage and translational inhibition (Bartel, 2004).

The miRNA precursor, pre-miRNA will fold into a stem-loop structure and DCL1 (DICER-LIKE1) will recognize the precursor to cut it into mature miRNA duplex (Bartel & Bartel 2003, Bartel 2004). Double stranded RNA is not stable in the cell and will be degraded in short time, but HEN1 can add methyl group at the 3'-end of the miRNA to protect it from degradation. The miRNAs will then be recognized by AGO1 (ARGONAUTE 1) and transported to the target, leading to mRNA cleavage or translational inhibition (Chapman & James C. Carrington, 2007).



(Modified from Chapman & James, 2007)

Figure 4. The pathways of miRNAs in *Arabidopsis thaliana*. Function of HEN1 is to methylate miRNAs, preventing them from degradation.

B. Motivation

We know that photomorphogenesis is a fast change in plant morphology. And it was reported that *ago1* mutant exhibited severe growth defects (Karen Bohmert, 1998). Also, the previous study suggested that at least 8 miRNAs were regulated by HY5 (Zhang *el al.*, 2011*)*. Also, HEN1 acts as a negative regulator of photomorphogenesis, which is also in part mediated by HY5 (Huang-Lung Tsai, 2014). So we wonder if there are more miRNAs that are light-regulated, and whether or not their targets will be cleaved upon light exposure through post-transcript regulation; hence, achieving photomorphogensis. We hope to find the miRNAs participating in photomorphogenic process, and further investigate their effects on development of *Arabidopsis* seedling.

C. Objectives

1. To find and validate the targeting of light regulated miRNAs and their predicted target mRNAs
2. To perform phenotypic studies for novel miRNA-target pairs that may be involve in photomorphogenesis.

D. Normalized sRNA NGS Data

(Conducted by Meng-Chun Lin and Huang-Lung Tsai in Wu's lab)

1. The number and percentage of light-regulated miRNAs of each category based on the selection principles (Figure 5), and the detailed normalized fold change (Figure 6) is exhibited, too.

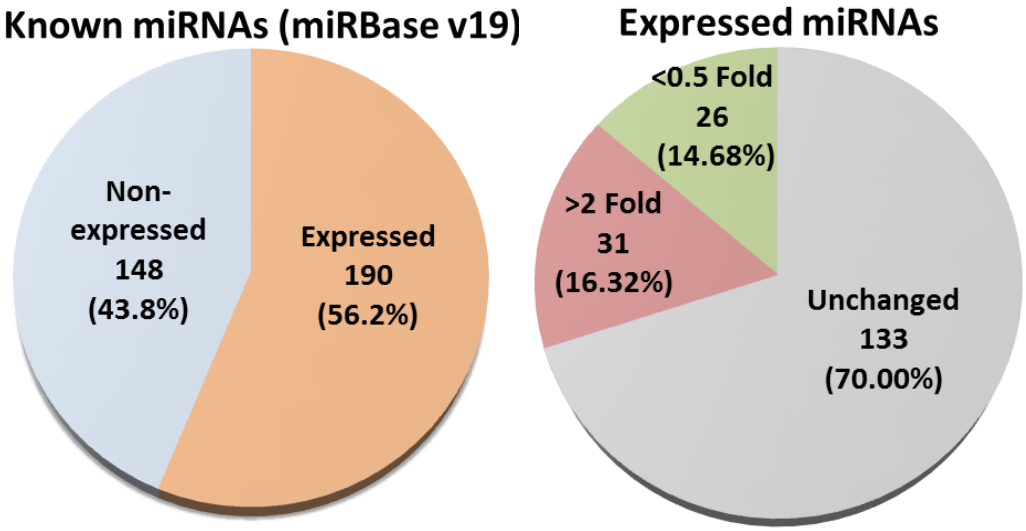


Figure 5. "Expressed" means "Read Count of an miRNA is more than 10 in at least one time point", known miRNAs is introduced from "miRBase v19". Fold change is normalized to 0hr (dark).

miRNA	0hr	1hr	3hr	6hr	12hr	24hr	TPM>10
miR396b	1	2.5510308	1.03845978	2.32369821	1.28841371	1.33907857	O
miR858a	1	4.77149086	2.2192403	5.07498628	1.3102633	1.85927296	O

Figure 6. The data shown are normalized to 0hr. Numbers indicate the fold changes of selected light-regulated miRNAs that are validated by northern blotting.

E. Comparison between the NGS Data of sRNA and mRNA and Introduction of Them

(Conducted by Meng-Chun Lin and Huang-Lung Tsai in Wu's lab)

1. Selected miRNA-target pairs with PCC

The form shows the targets which miRNAs may regulate and the correlation between each other (Figure 7). The correlation value which is lower than – 0.5 indicates that the miRNAs are related to targets more possibly. For example, the correlation of *MIR396A* and its target, *At2g36400*, is – 0.55554, so we can infer that miR396a has big chance to degrade *GRF3*. Moreover, miR858a may be related to the degradation of *At4g09460* according to the correlation value.

Light-regulated miRNA and selected targets			
miRNA	Target	Gene	PCC
miR396b	*At1g80060.1*	UBQ-like superfamily protein	-0.68884
miR396a	*At2g36400.1*	*GRF3*	-0.55554
miR858a	*At4g09460.1*	*MYB6*	-0.544305
miR858a	*At5g05800.2*	Unknown	-0.84417

(Pearson correlation coefficient(PCC) = $\sum_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y}) / \sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2 \cdot \sum_{i=1}^{n}(y_i-\bar{y})^2}$)

Figure 7. Selected miRNA-target pairs with Pearson correlation coefficient shown. The equation of it is also exhibited.

2. miR396-target pairs

a. NGS data

The change of *MIR396A* and *MIR396B* is opposite, and *MIR396A* increases while *MIR396B* reduces. They may be complementary relationship. *At1g80060*, of which the quantity stays low, may be slightly regulated by mi396s. *At4g37740*, *At2g36400* and *At5g53660* are *GRF*s (Growth-Regulating Factor) (Figure 8). They all decrease immediately after 1hr white light exposure, which may result from the increasing of miR396b. In the following time, The amount of the *GRF*s stays low, which has the chance that miR396s are functional throughout (Figure 9).

Gene	Description
At1g80060	A member of UBQ-like superfamily protein
At4g37740	Growth-Regulating Factor 2
At2g36400	Growth-Regulating Factor 3
At5g53660	Growth-Regulating Factor 7

Figure 8. The description and function of each selected target.

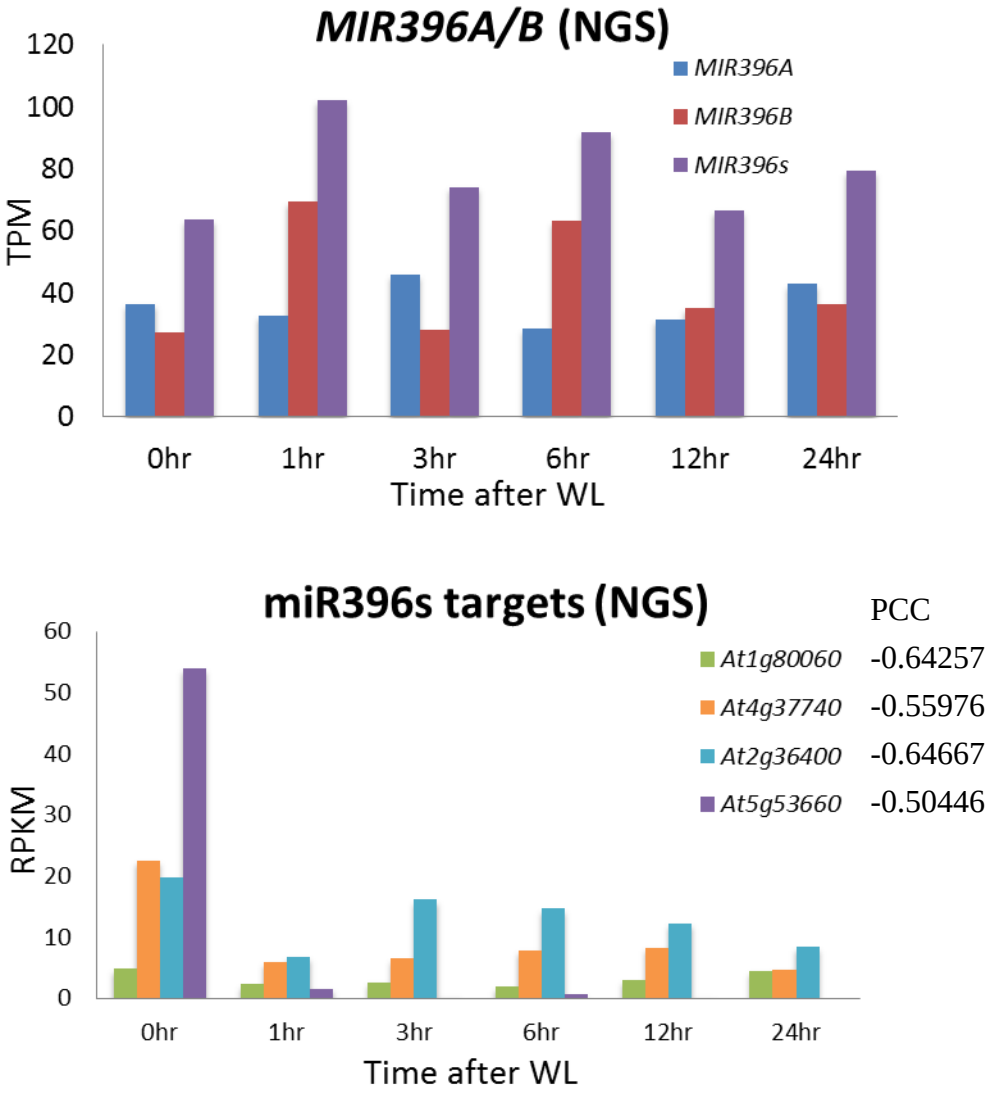


Figure 9. The NGS data of *MIR396*A/B and their targets. *MIR396*s indicates the sum of *MIR396A* and *MIR396B.* X-axis stands for the time after white light exposure. Y-axis in upper figure is the read counts which is detected by machine, and the other is the RPKM (Reads Per Kilo base per Million reads), Also, Pearson correlation coefficients are shown which are calculated on the basis of *MIR396*s.

(Conducted by Meng-Chun Lin and Huang-Lung Tsai in Wu's lab)

b. Introduction

The cleavage sites, the hybrids of miR396s and their targets, *At1g80060*/ *At4g37740*/ *At2g36400*/ *At5g53660*, are shown respectively (Figure 10, 11).

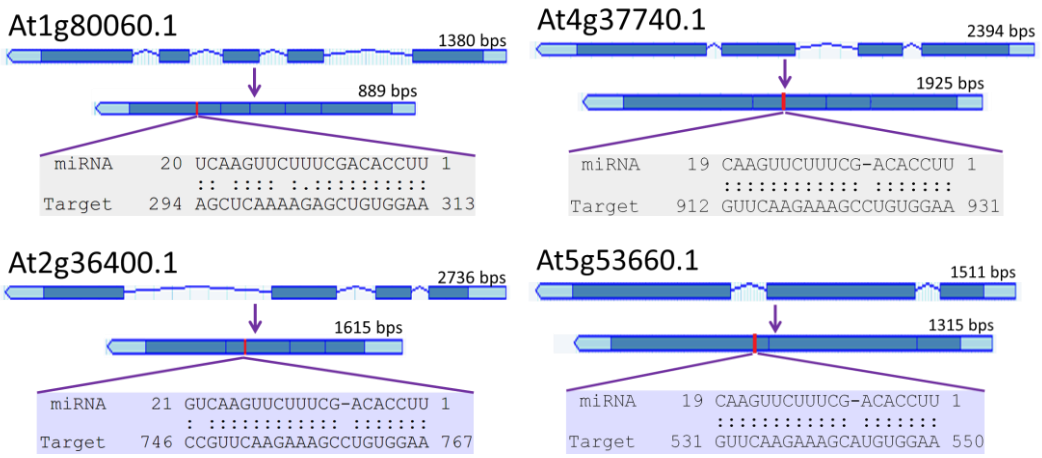


Figure 10. The cleavage sites of miR396 targets and the combinations of miR396-target pairs (Take miR396a for example), *At1g80060*/ *At4g37740*/ *At2g36400*/ *At5g53660*. The upper bars stand for pre-mRNA, while the lower bars stand for mRNA.

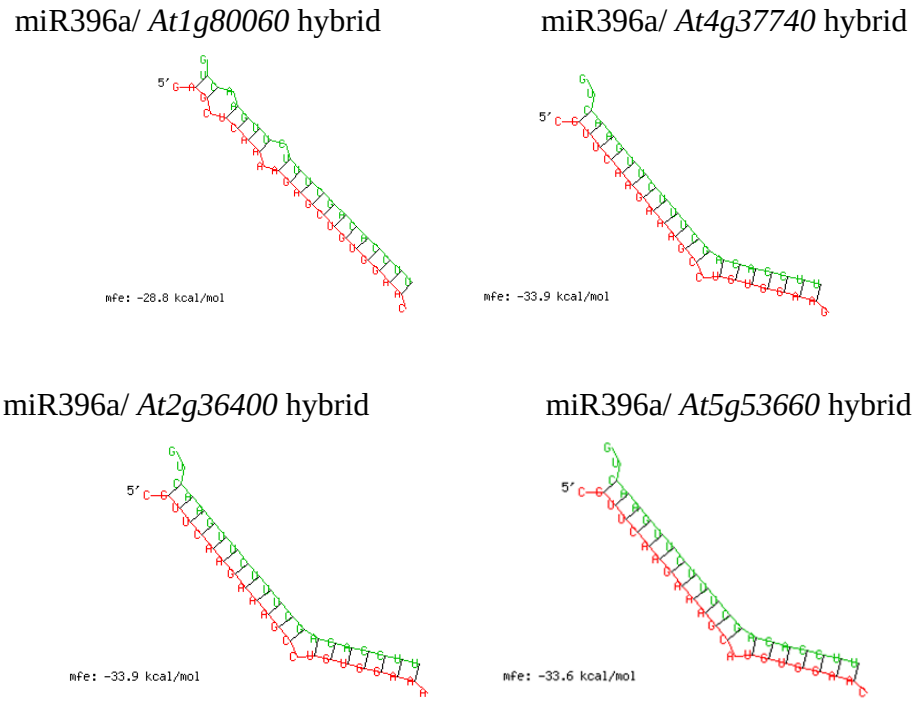


Figure 11. The hybrids of miR396s and their targets (Take miR396a for example), *At1g80060*, *At4g37740*, *At2g36400*, *At5g53660*. The green sequences stand for the miR396a and the red sequences stand for the targets. The free energy (mfe) for each miR396a-target pair was listed.

2. miR858-target pairs

a. NGS data

At4g09460 and *At5g05800* are the targets we choose (Figure 12). Both the targets reduce after 1 hr, and increase slowly after 3 hr white light exposure. The quantity of *MIR858*s has a transient accumulation, keeping the targets in a low level in the following time. But it needs more evidence. The change pattern of *MIR858*s roughly corresponds to its targets, except the time point: 6hr. So, there might be another reason resulting in this case. (Figure 13)

Gene	Description
At5g05800	Unknown
At4g09460	MYB6 DNA-binding protein

Figure 12. The description of each selected target.

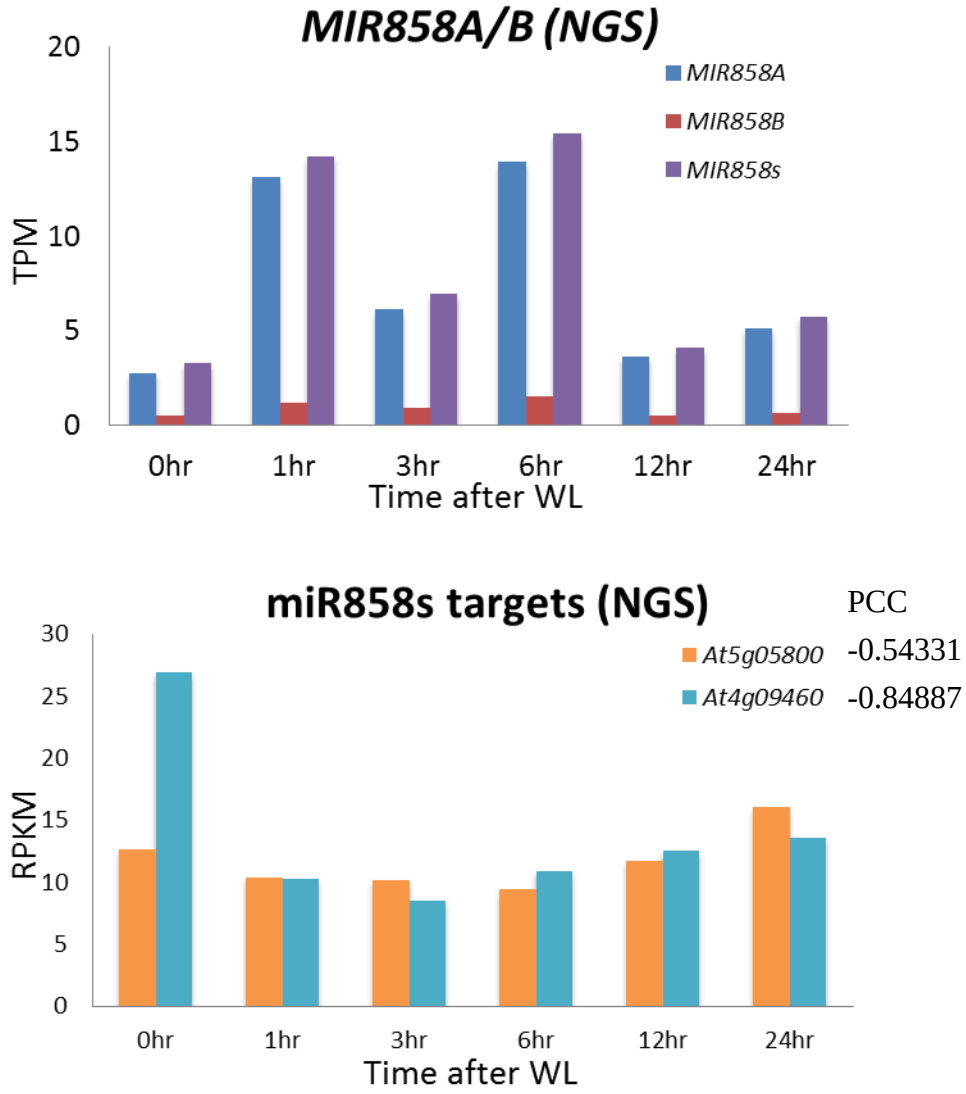


Figure 13. The NGS data of *MIR858*A/B and their targets. *MIR858*s indicate the sum of *MIR858A* and *MIR858B*. X-axis stands for the time after white light exposure. Y-axis in upper figure is the read counts which is detected by machine, and the other is the RPKM (Reads Per Kilo base per Million reads). Also, Pearson correlation coefficients are shown which are calculated on the basis of *MIR858*s.

(Conducted by Meng-Chun Lin and Huang-Lung Tsai in Wu's lab)

b. Introduction

The cleavage sites, the hybrids of miR858s and their targets, *At4g09460*/ *At5g05800*, are shown (Figure 14, 15).

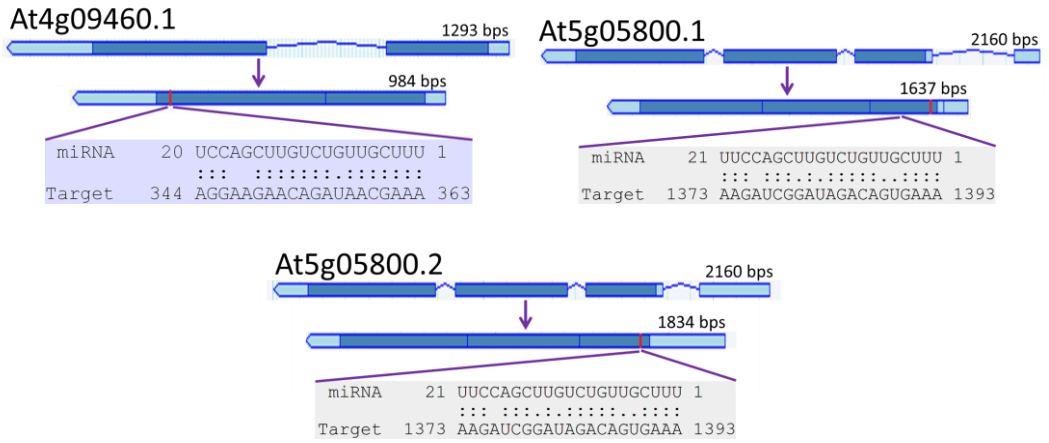


Figure 14. The cleavage sites of miR858 targets and the combinations of miR858-target pairs (Take miR858a for example), *At4g09460* and *At5g05800*. The upper bars stand for pre-mRNA, while the lower bars stand for mRNA.

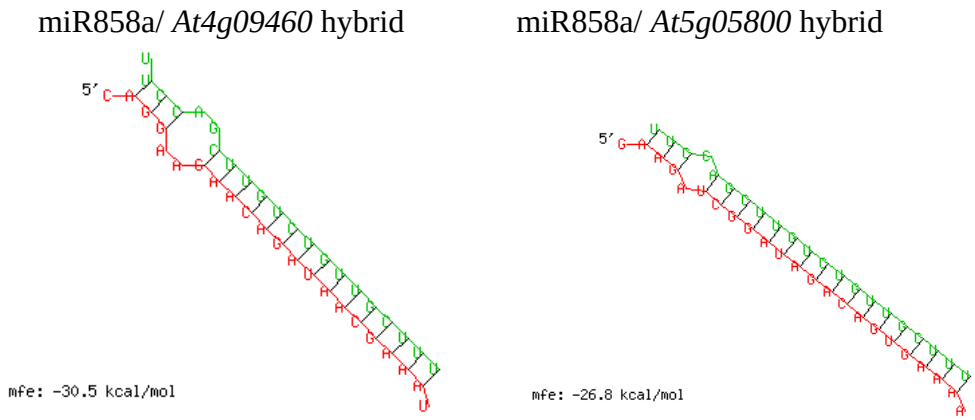
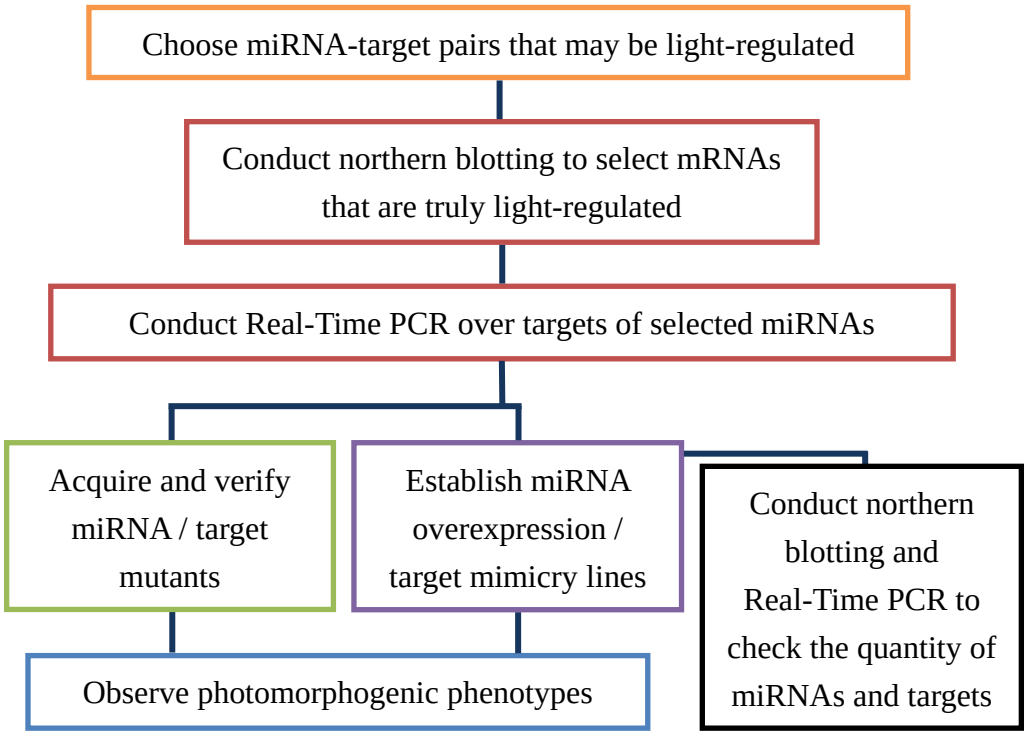


Figure 15. The hybrids of miR858s and their targets (Take miR858a for example), *At4g09460*, *At1g05800*. The green sequences stand for the miR858a and the red sequences stand for the targtets. The free energy (mfe) for each miR858-target pair was listed.

Experimental Design and Procedure

A. Research Flow



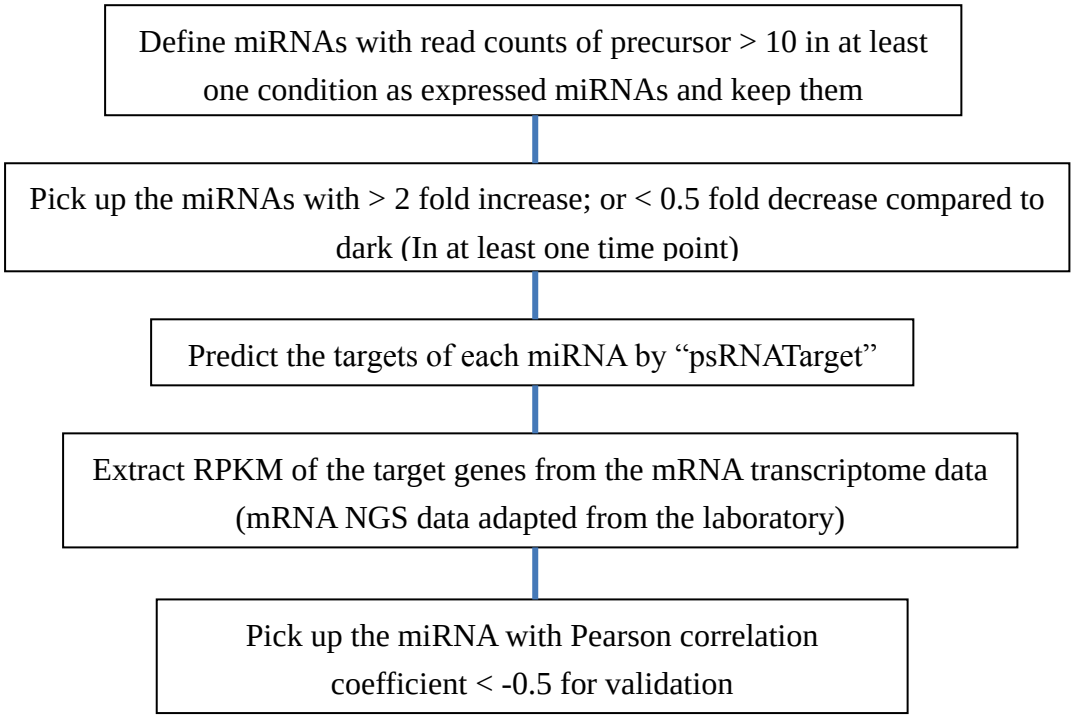
The flow diagram above is for our entire research. It can be separated to six parts:

1. Choose the miRNA-target pair candidates. **(Orange)**
2. Conduct northern blotting and Real-Time PCR to verify the miRNA-target pairs are truly light-regulated. **(Red)**
3. Acquire and verify the mutants of the miRNAs / targets. **(Green)**
4. Establish the miRNA overexpression / target mimicry lines. **(Purple)**
5. Observe the photomorphogenic phenotypes. **(Blue)**
6. Confirm the relationship of miRNA-targets by conducting northern blotting and Real-Time PCR over the miRNA overexpression / target mimicry lines. **(Black)**

B. Choose the miRNA-Target Pair Candidates

1. The acquisition and analyses of NGS data were conducted by Meng-Chun Lin and Huang-Lung Tsai in Wu's lab

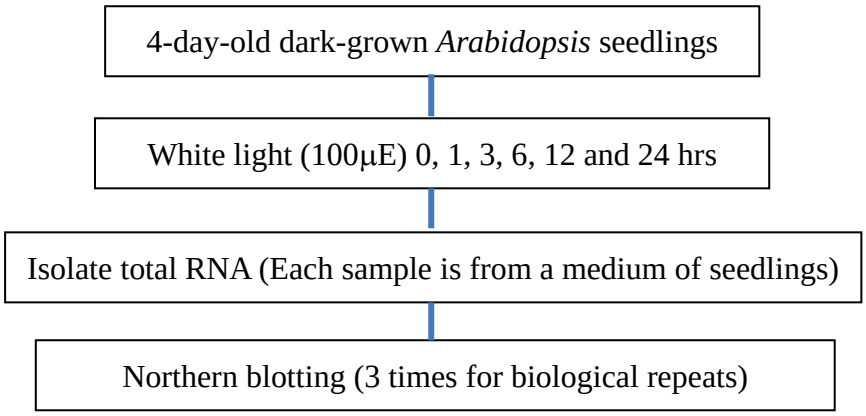
2. The selection flow done by Meng-Chun Lin and Huang-Lung Tsai in Wu's lab



C. Conduct Northern Blotting and Real-Time PCR to Verify miRNA-Target Pairs.

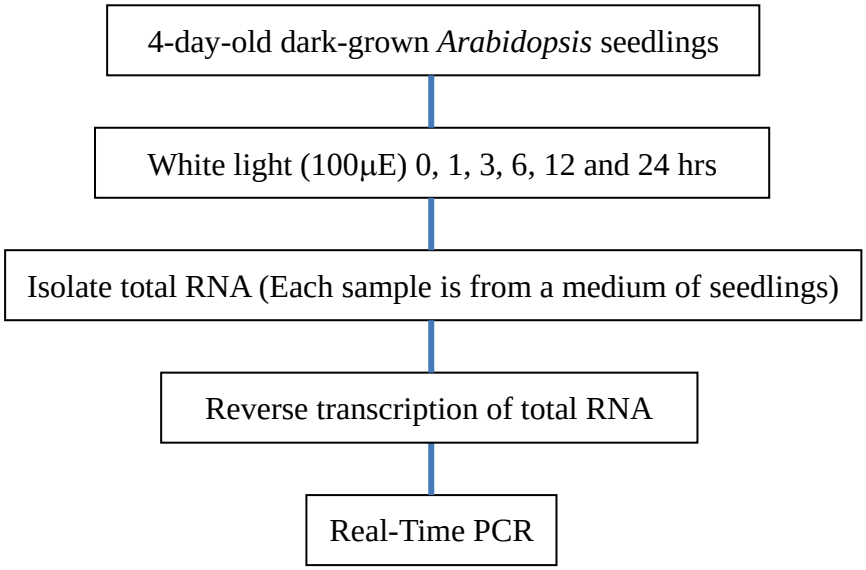
1. Conduct northern blotting

Although we get some evidence supporting the miRNAs which we selected are light-regulated from the NGS analysis, we needs more biological repeats to support their light regulation. Northern blotting is a general and practical method in validating RNA expression. By this method, we can confirm the expression change of the light-regulated miRNAs in NGS data.



2. Conduct Real-Time PCR for the targets of the selected miRNAs

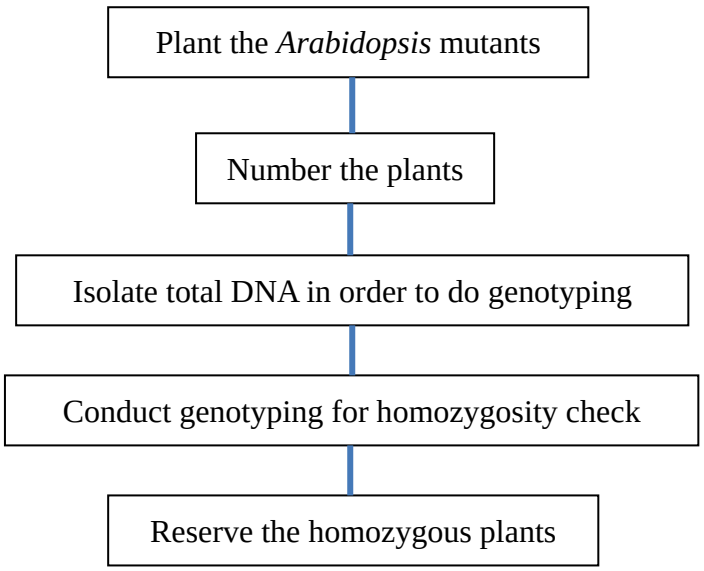
Real-Time PCR is a method to know the precise amount of genes through detection of fluorescence intensities. So we used Real-Time PCR to validate the results of miRNA targets showing obvious anti-correlation ($r < -0.5$) with their corresponding miRNAs.. Pearson correlation coefficient is based on the sum quantity of miRNA family. Also, the validated targets from other literature, e.g., *GRF*s, are also considered. (Jones-Rhoades, M.W., Bartel, D.P. ,2004))



D. Acquire and Verify the Mutants of the miRNAs / Targets.

1. Acquire and verify the homozygous *Arebidopsis* mutants

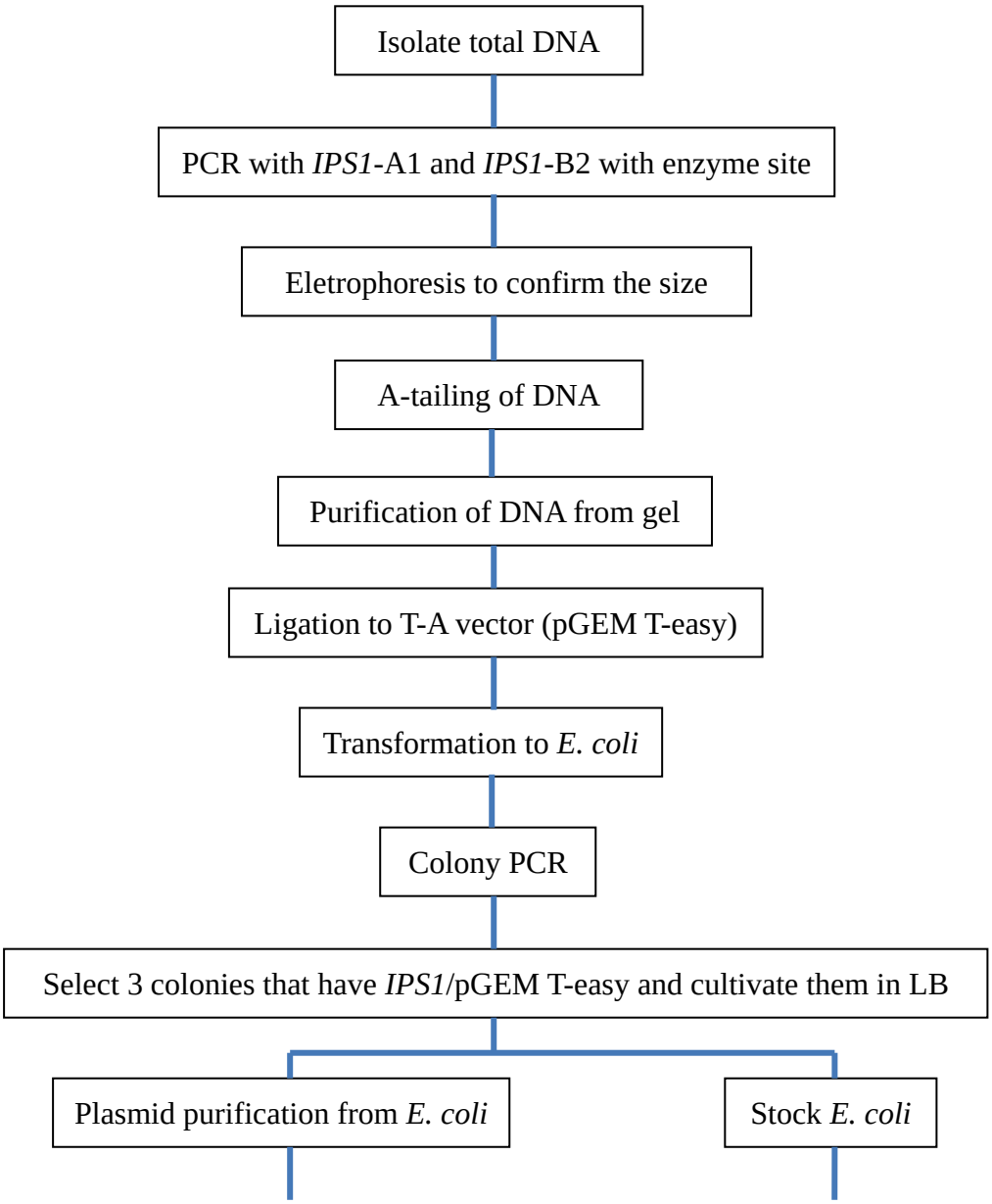
The single mutants, *grf2* and *grf3*, for investigation are from Dr. Jeong Hoe Kim's lab, and the mutants above are Ws background**.** In addition, *grf7-1*(SAIL_1256_F08) are from Dr. Shinozaki's lab, RIKEN, and the mutants are Col background. The other mutants which we use are from Salk Institute. Repeating first four steps until we get homozygous.

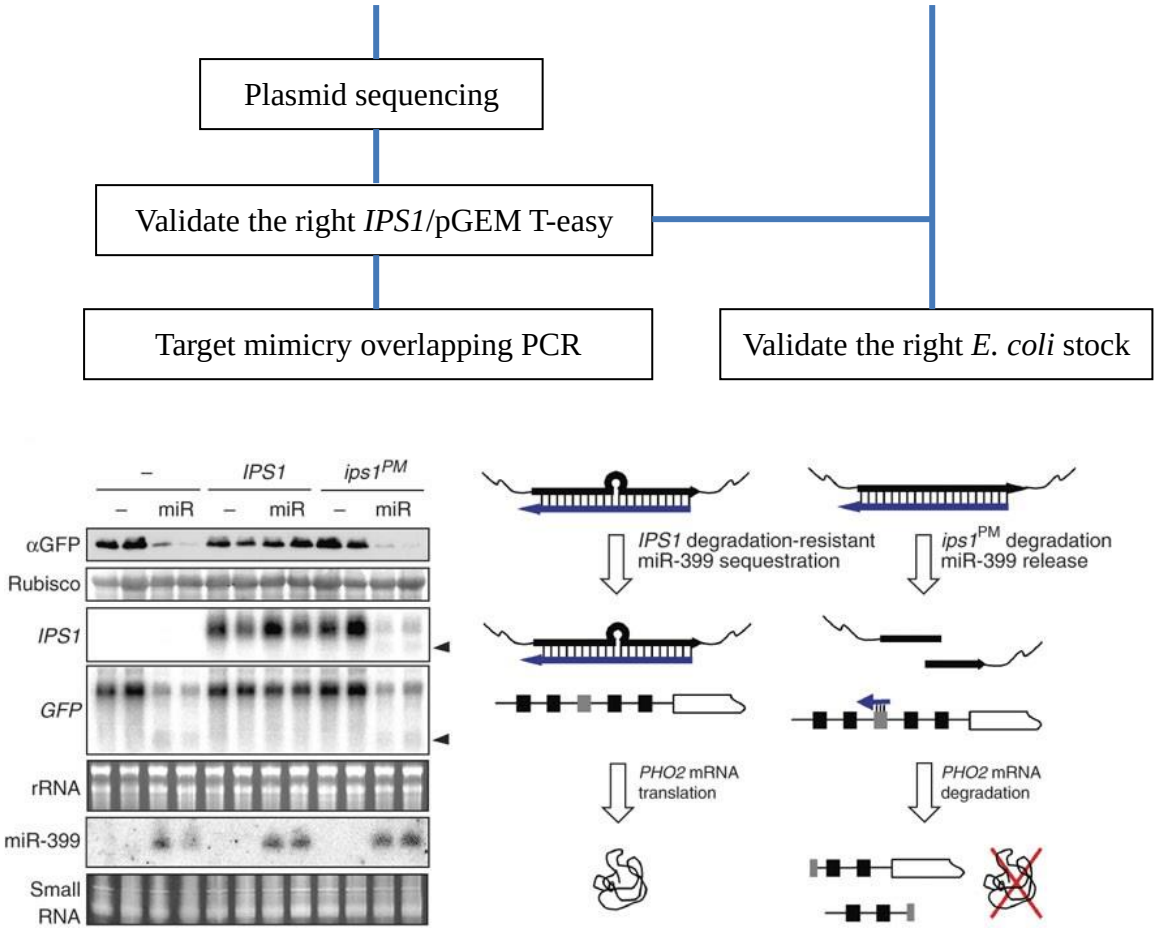


E. Establish miRNA Overexpression and Target Mimicry Lines.

1. Build the target mimicry inserts

This part includes two sections, building *IPS1*/pGEM T-easy construct and target mimicry overlapping PCR. Target mimicry is on the basis of miRNA binding sites which is non-perfect match (Figure 16). Overlapping PCR is the way that we add the non-perfect match sequences (Figure 17). The PCR products of *IPS1* with enzyme-cut sites (Forward: *Sal*I; Reverse: *Sac*I) and will be used as templates of 2° PCR. A-tailing is the preparation for ligation to T-A vector (pGEM T-easy). We can get purified *IPS1*/pGEM T-easy through the following steps. Colony PCR is to check whether the plasmids have insert of our interest (in here means *IPS1*).





(Jose' Manuel Franco-Zorrilla, 2007)

Figure 16. *ips1*^{PM} is degraded by miRNA immediately, and releases RISC. If the sequences of miRNA binding-site is non-perfect match, miR399 will stick to *IPS1* losing the function of degrading the other targets.

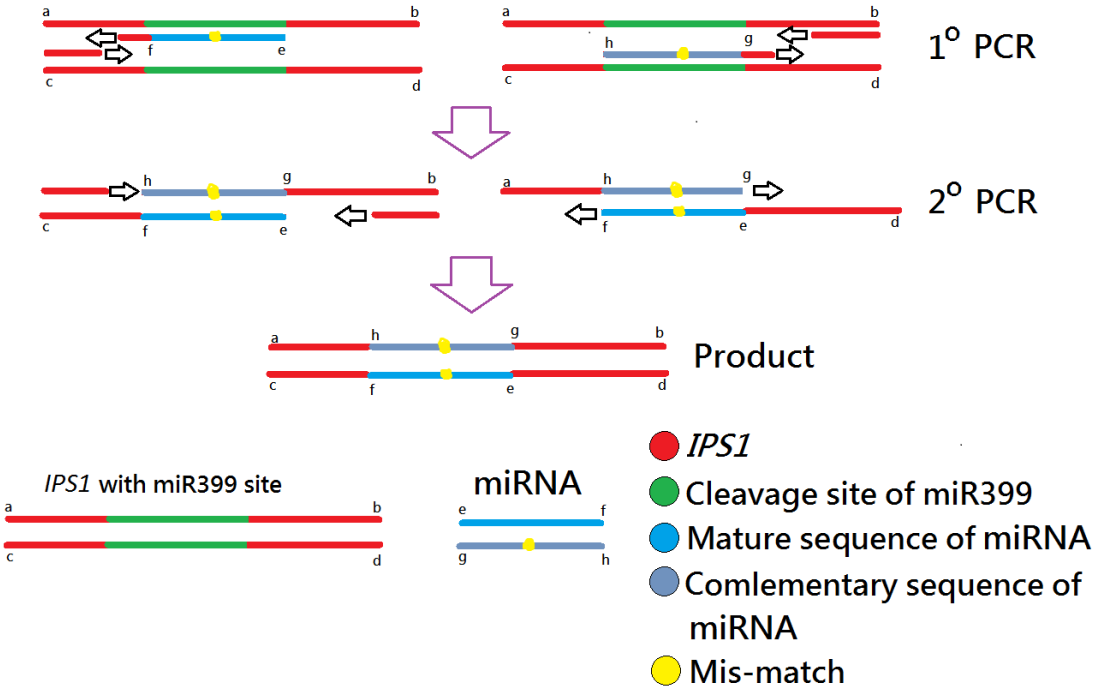


Figure 17. The method we design to get target mimicry is overlapping PCR. 1° PCR is separated to two sections conducted with two sets of primers. PCR product of one section contains upstream sequences of *IPS1* and the miRNA sequences (non-perfect match), and the other contains downstream sequences of *IPS1* and the miRNA sequences (non-perfect match). We then mixed the two product as templates for 2° PCR.

2. Establish the target mimicry and overexpression lines

This part includes seven sections (Figure 18, 19):

a. Building *MIMRNA*/pGEM T-easy (for target mimicry line) and *35S::MIRNA*/pGEM T-easy (for miRNA overexpression line)

b. *Sal*I and *Sac*I digestion

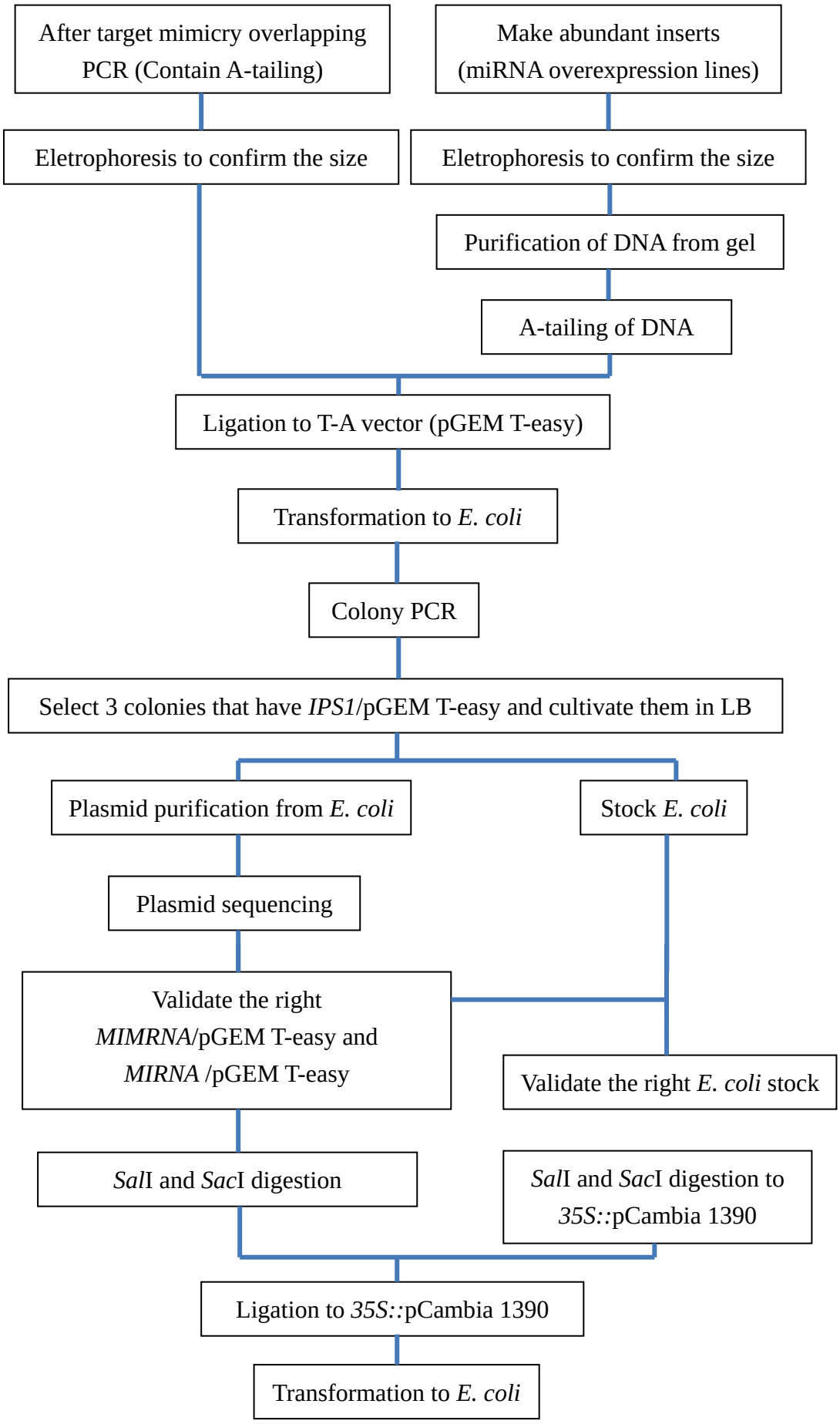
c. Building *35S::MIMRNA*/ pCambia 1390 (target mimicry line) and *35S::miRNA* / pCambia 1390 (miRNA overexpression line)

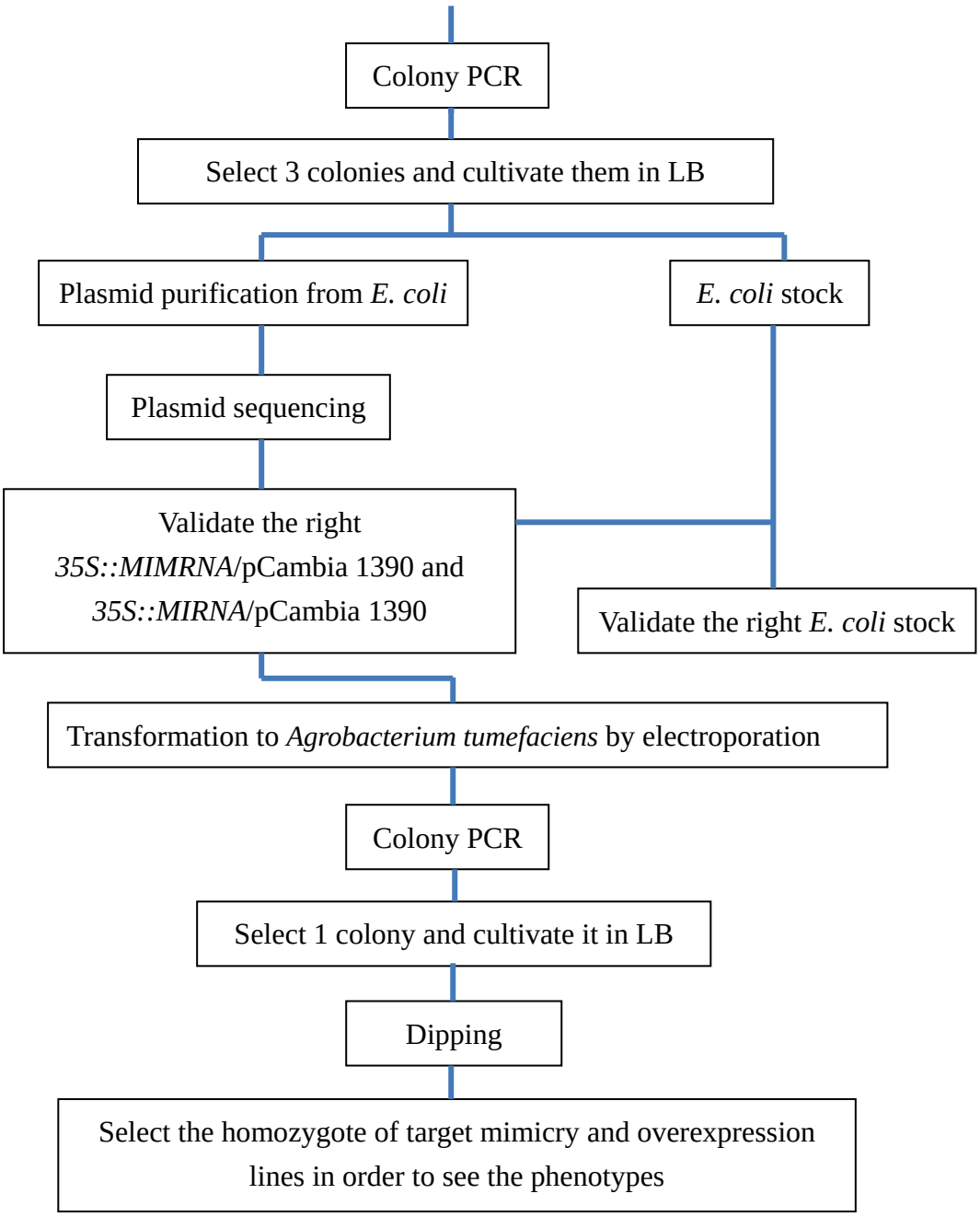
d. Transformation to *Agrobacterium tumefaciens*

e. Transformation to plant by floral dipping

f. Select homozygotes of target mimicry and overexpression lines

g. Verification of the overexpression and target mimicry lines by northern blotting


After target mimicry overlapping PCR (Contain A-tailing)
Make abundant inserts (miRNA overexpression lines)
Eletrophoresis to confirm the size
Eletrophoresis to confirm the size
Purification of DNA from gel
A-tailing of DNA
Ligation to T-A vector (pGEM T-easy)
Transformation to E. coli
Colony PCR
Select 3 colonies that have IPS1/pGEM T-easy and cultivate them in LB
Plasmid purification from E. coli
Stock E. coli
Plasmid sequencing
Validate the right MIMRNA/pGEM T-easy and MIRNA /pGEM T-easy
Validate the right E. coli stock
SalI and SacI digestion
SalI and SacI digestion to 35S::pCambia 1390
Ligation to 35S::pCambia 1390
Transformation to E. coli


Colony PCR
Select 3 colonies and cultivate them in LB
Plasmid purification from *E. coli*
E. coli stock
Plasmid sequencing
Validate the right *35S::MIMRNA*/pCambia 1390 and *35S::MIRNA*/pCambia 1390
Validate the right *E. coli* stock
Transformation to *Agrobacterium tumefaciens* by electroporation
Colony PCR
Select 1 colony and cultivate it in LB
Dipping
Select the homozygote of target mimicry and overexpression lines in order to see the phenotypes

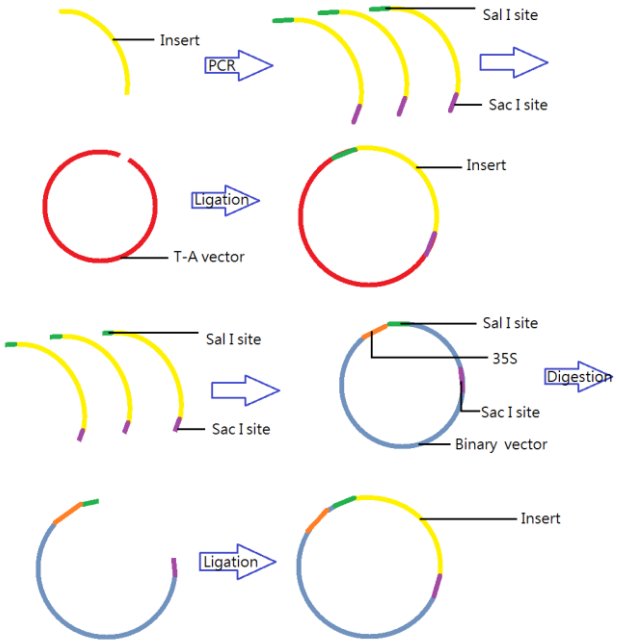


Figure 18. The brief diagram is the flow chart of cloning an insert to the binary vector (*35S::*pCambia 1390). While building the target mimicry constructs, the first PCR step is skipped because we already have the insert digested from pGEMT-easy.

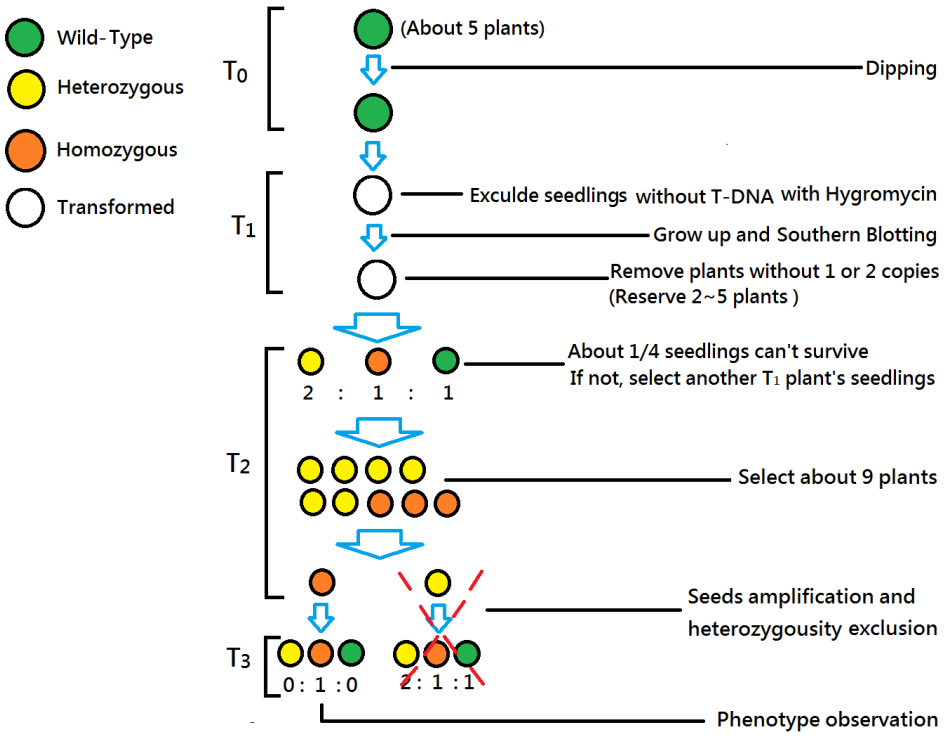
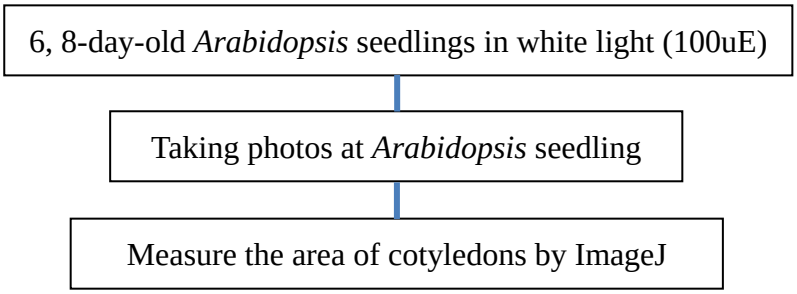


Figure 19. The flow chart of selecting homozygotes when we do not know the insertion site. The method is based on Mendel's law. Nontheless, instead of getting T-DNA allele, the plants we observe are with T-DNAs will not segregate.

F. Observe the Phenotypes

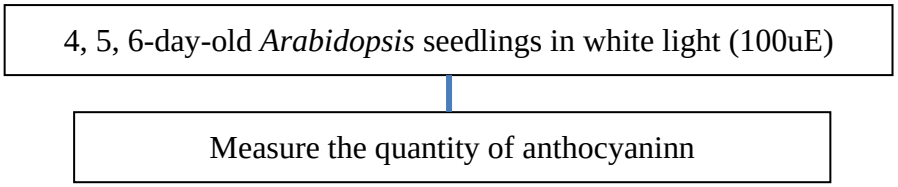
1. Measurement of the area of cotyledons

Cotyledons of Col-0 seedlings will be full opened on about 6 days after germination (Douglas, 2001). But germination does not happen as soon as white light exposure, and the cotyledons are too small to identify the difference before 4 days in white light, so we select 6, 8-day-old *Arabidopsis* seedlings to observe. By this way, we can investigate the development rate and the size of the cotyledons.



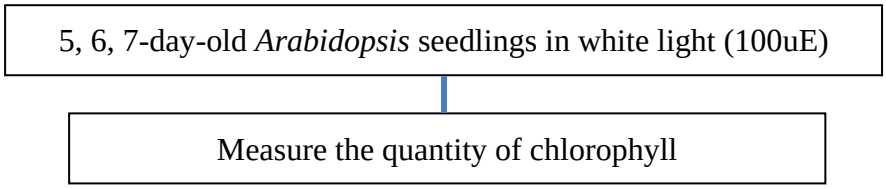
2. Measurement of the quantity of anthocyanin

3-day-old *Arabidopsis* seedlings are too small to collect, and previous research select 5-day-old seedlings to measure the anthocyanins content (Chiung-swey Joanne Chang, 2008). In our present results, anthocyanins contents in both wild type and mutants are similar in 6-day-old seedlings. So we chose 4, 5, 6-day-old *Arabidopsis* seedlings to observe.



3. Measurement of the quantity of chlorophyll

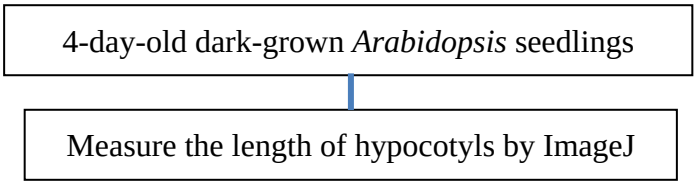
Previous research use 6-day-old seedlings to measure the chlorophyll content (Chiung-swey Joanne Chang, 2008), and in our previous results, chlorophyll contents in both wild type and mutants are similar in 4, 5-day-old seedlings but have apparent difference after 6 days. Thus, we also want to know the situation of the seedlings after 7 days in white light.



4. Measurement of the length of hypocotyl

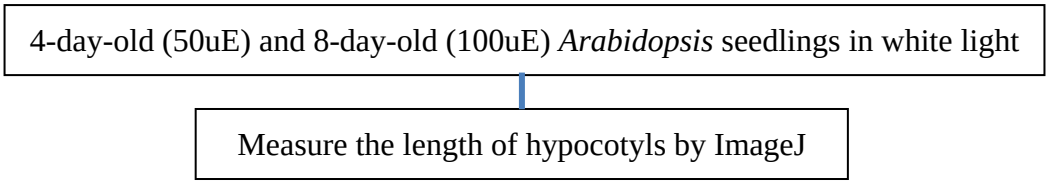
a. Dark-grown seedlings

We want to know if the mutated gene affect the rate of hypocotyl elongation. The length of hypocotyls of 4-day-old dark-grown *Arabidopsis* seedlings are truly long in our test while more day in dark could give rise to other variables. So, 4-day-old seedlings are choosed.



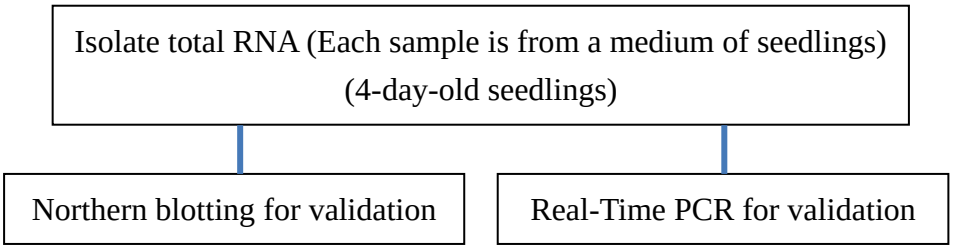
b. Light-grown seedlings

4-day-old seedlings are observed, which is used to know the rate of hypocotyl elongation. But if we put them 100uE white light, the hypocotyls will be to short to recognize the difference. 8-day-old seedlings are choosed to know whether the selected targets have a role in the length of fully lengthened hypocotyls.



G. Confirm the Relationship of miRNA-targets by Conducting Northern Blotting and Real-Time PCR over the miRNA Overexpression / Target Mimicry Lines

1. Check the quantity of the miRNA and selected targets in the miRNA Overexpression / Target Mimicry Lines



Even if there are visible phenotypes, we are not sure of the in-enviroment of the seedlings, the quantity of miRNA and the targets. Thus, the validation is needed. Northern blotting is for checking the quantity of the miRNA, while Real-Time PCR is for checking the amounts of the targets. The samples are 4-day dark-grown seedlings after 0hr/ 1hr white light exposure because the quantity of targets has rapid change from 0hr to 1hr. With the results of this experiment, we can further verify the cleavage events.

Results

A. Individual Results of Two Selected Sets of miRNA-target pairs

miRNA396a/b, miRNA858a/b are selected after NGS analysis and northern blotting. The result below is the arrangement of miRNA data, including NGS, northern blotting, and Real-Time PCR for its targets (of which Pearson correlation coefficients with the sum of miR396 or miR858 family are less than -0.5). We can compare the figures and check the relationship between miRNAs and targets. Finally, we can ensure that miR396s and miR858s act as important roles in photomorphogenesis.

1. miRNA396

a. Comparison between miR396s and targets

The quantity of miR396s decreases stably in the results of northern blotting (Figure 20), and the fold changes of the targets increase after 1 hr white light exposure generally except *At5g53660* (Figure 21). So, miR396s may regulate them in photomorphogenesis. However, the inhibited accumulation of *At5g53660* may be caused by other factors. Expression of the targets was decreased at first of white light, and eventually increased (Figure 21). Still, the miR396-target pairs truly show anti-correlation. So, it is possible that miR396s regulate a member of UBQ-like superfamily protein and Growth-Regulating Factor 2/ 3/ 7.

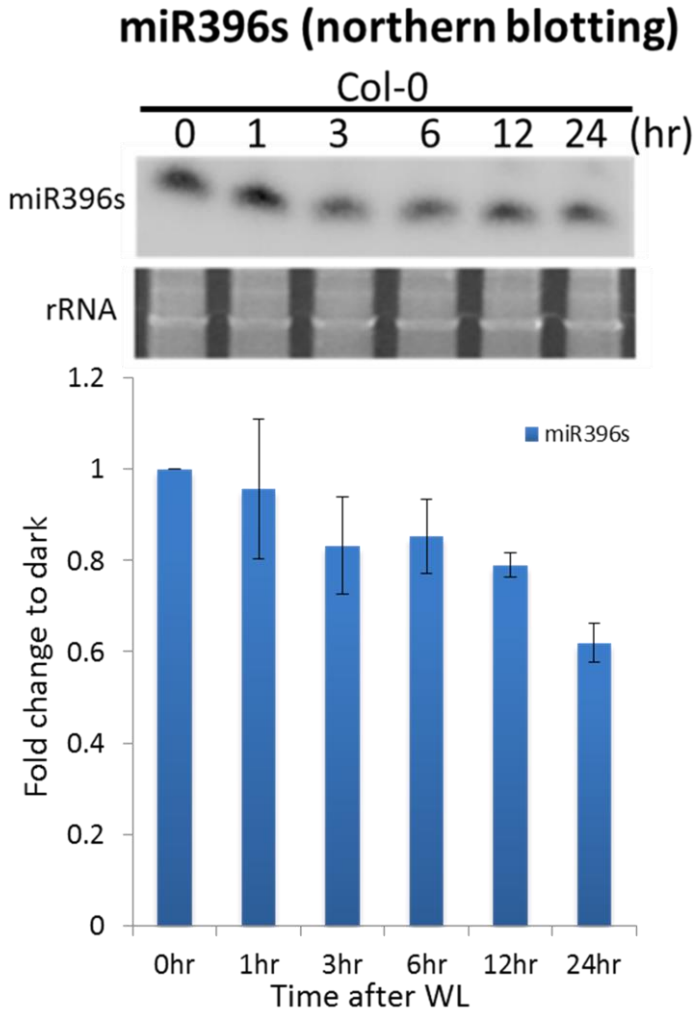


Figure 20. The result of northern blotting for miR396s. Fold change to dark is used to normalize miR396s expression of all six time points. The error bar is from 3 biological repeats. The amount of RNA for each sample is 20μg. rRNA (ribosomal RNA) is used as loading control. X-axis stands for time after white light exposure, and Y-axis represents the fold change compared to dark condition. Experiments involving isotope was conducted by Meng-Chun Lin.

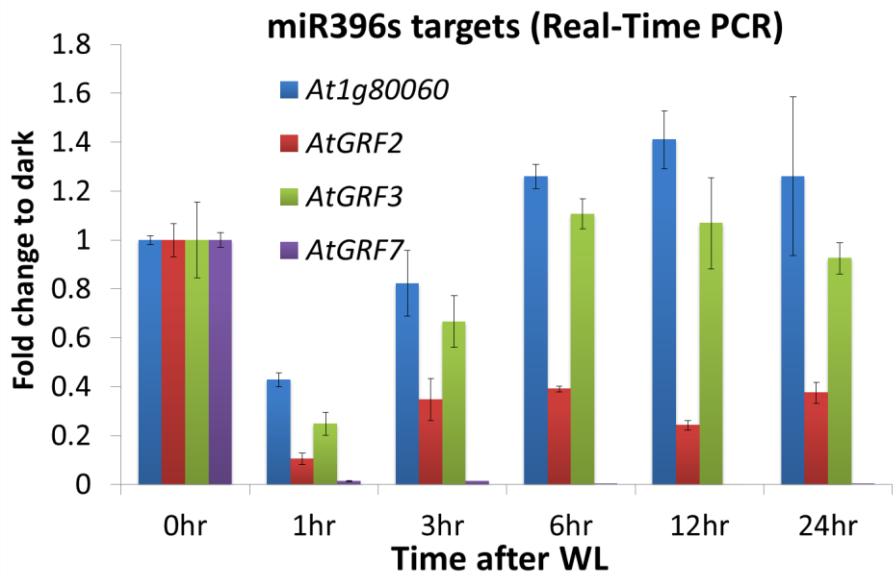
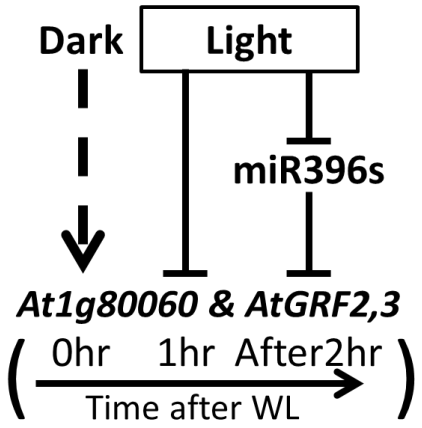
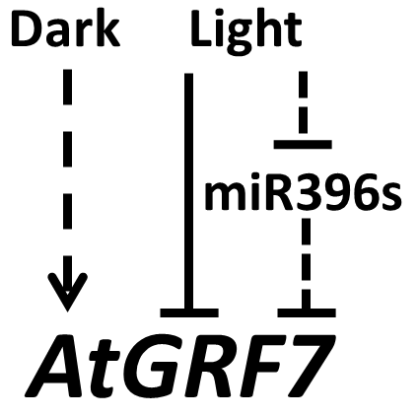


Figure 21. One of the three biological repeats of Real-Time PCR for the selected targets, *At1g80060*, *At4g37740*, *At2g36400*, *At5g53660*. The error bar is from 3 technical repeats. X-axis stands for the time after white light exposure, and Y-axis represents the fold change compared to dark condition.

b. Proposed model



We can see that miR396s expression pattern corresponds to the expression patterns of the selected *At1g80060* and *AtGRF2, 3* after white light exposure. Also we find that there is immediate decrease of the targets after 1 hour white light exposure, so there should be a promoter making the targets highly express in dark. Upon exposed to white light, light can induce some factors to switch off this process. Afterwards, miR396s take the main role regulating the targets,



We can see that miR396s expression pattern does not quite correspond to the expression patterns of *AtGRF7* after white light exposure. Also we find that there is immediate decrease of the targets after 1 hour white light exposure and the amount of *AtGRF7* remains very low compared with that in dark, so there should be a promoter making the targets highly express in dark. Upon exposed to white light, light can induce some factors to switch off this process. And this inhibition may means a lot more important than miR396s physiologically.

2. miRNA858

a. Comparison between miR858s and targets

The quantity of miR858s first increases and then decreases stably in the results of northern blotting (Figure 22), and the fold changes of the targets decrease after 1 hr white light exposure generally (Figure23). So, miR858s may regulate them in photomorphogenesis. Then the accumulation of miR858s corresponds to the increase of the targets. The anti-correlation between the miR858 and target are obvious. So, it is possible that miR858s regulate MYB6 DNA-binding protein and the unknown protein *At5g05800*.

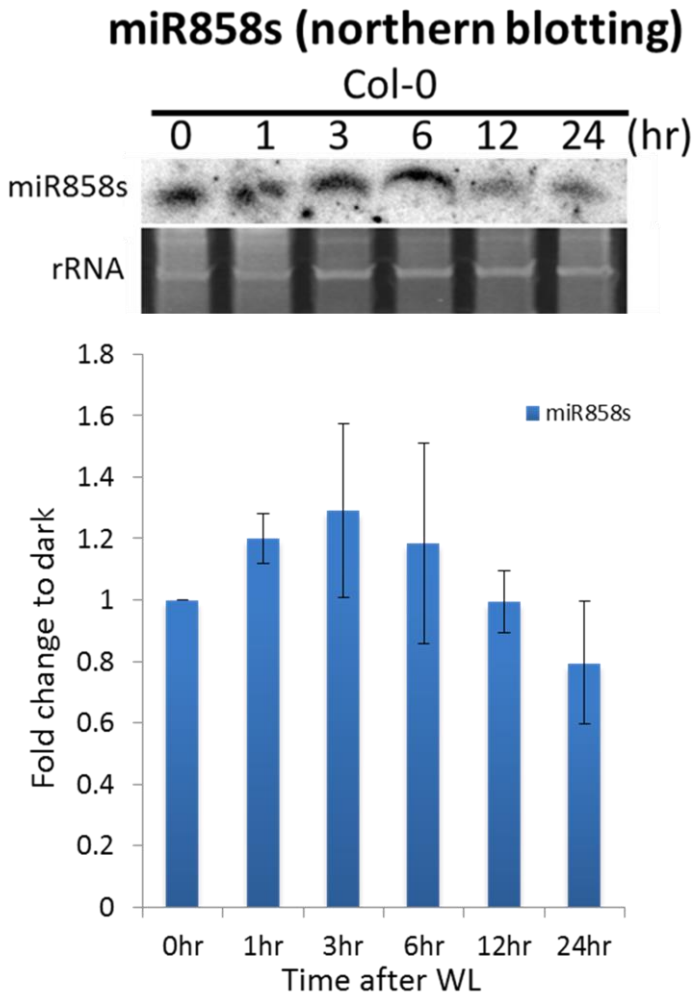


Figure 22. The result of northern blotting for miR858s. Fold change to dark is used to normalize miR858s expression of all six time points. The error bar is from 3 biological repeats. The amount of transfer RNA for each sample is 20μg. rRNA (ribosomal RNA) is used as loading control. X-axis stands for time after white light exposure, and Y-axis represents the fold change compared to dark condition. Experiments involving isotope was conducted by Meng-Chun Lin.

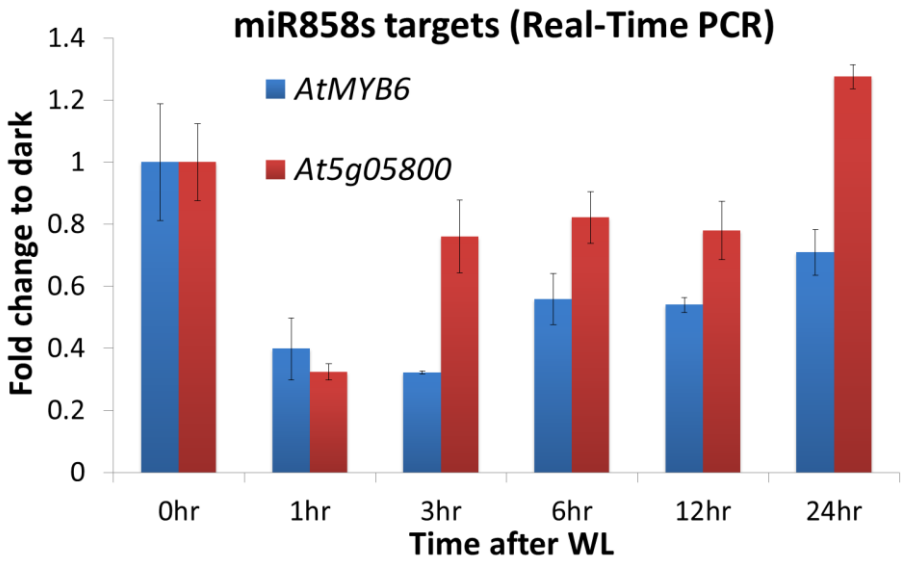
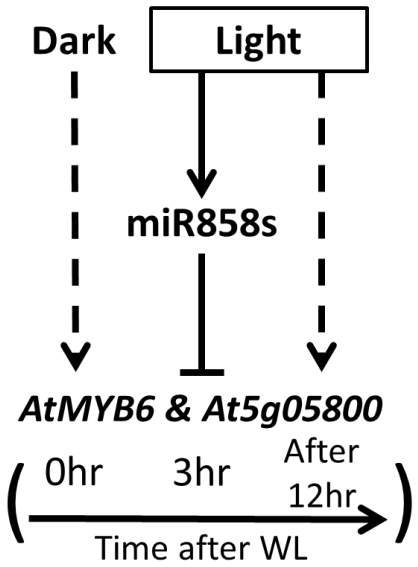


Figure 23. One of the three biological repeats of Real-Time PCR for the selected targets, *At4g09460*, *At5g05800*. The error bar is from 3 technical repeats. X-axis stands for the time after white light exposure, and Y-axis represents the fold change compared to dark condition.

b. Proposed model



We can see that the expression patterns of miR858s and the targets, AtMYB6 and At5g05800, are corresponding. It could means that miR858s take the main regulator of the decrease of the targets.

B. Results of Observing the Phenotypes of Mutants of miRNAs/ miRNAs Targets

1. miR396

a. The mutants used in observing the phenotypes (Figure 24)

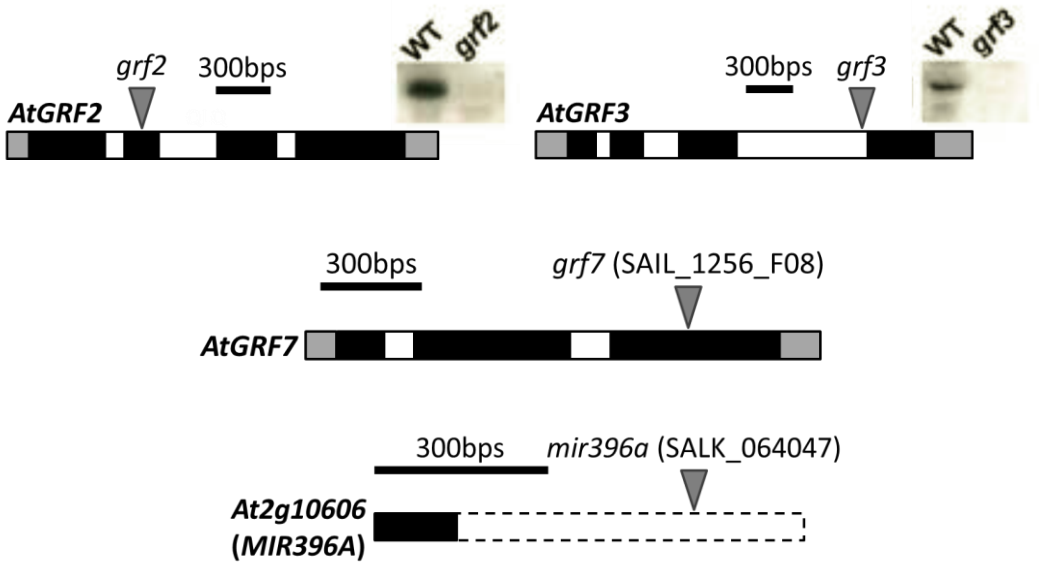


Figure 24. Schematic gene structures showing T-DNA insertion sites are marked with arrowheads. Black, gray, white bars and dotted border indicate exons, non-translated regions, introns and non-transcribed region respectively. (Jeong Hoe Kim et al, 2003)

b. Anthocyanin content

The biggest difference of anthocyanin content between wild types (Col-0 and Ws) and mutants exists after four days in white light. While *grf7* and *mir396a* have more anthocyanin content from 4 to 5 days after white light, only *grf3* shows less anthocyanin content then wild type. GRF2 seems to have no big influence in the anthocyanin biosynthesis. We conclude that GRF7 (Growth-Regulating Factor 7) and miR396a negatively regulate the biosynthesis of anthocyanin to white light, while GRF3 (Growth-Regulating Factor 3) plays the opposite role (Figure 25).

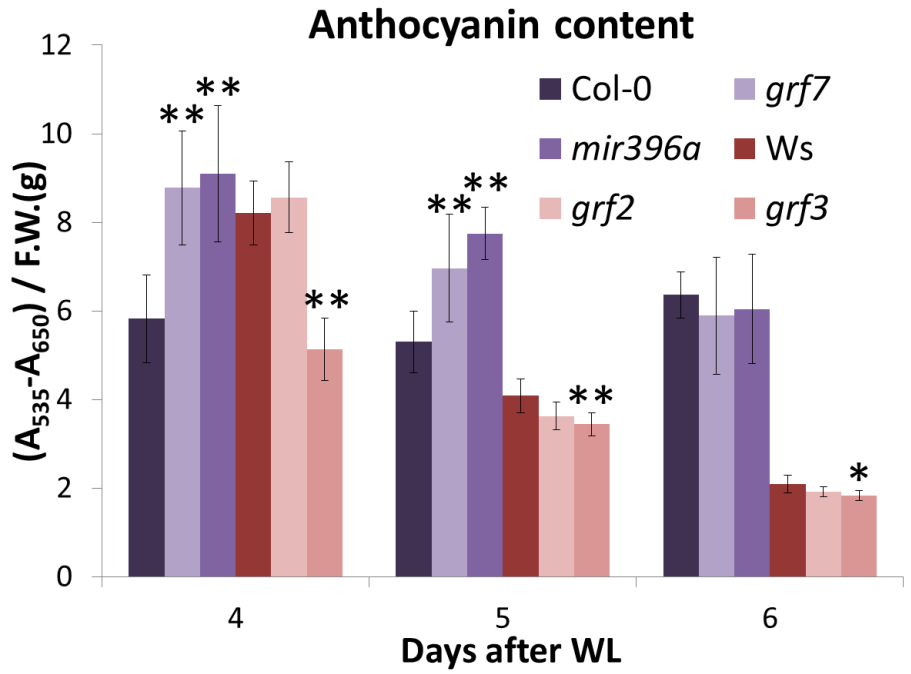


Figure 25. *grf7* and *mir396a* have more anthocyanin content after 4 and 5 days, while *grf3* have less anthocyanin content after 4 to 6 days in WL(White Light). The error bars indicate mean ± STD from 4-6 biological repeats. Media used are with 1% sucrose to increase anthocyanin. "*" indicates P<0.05; "**" indicates P<0.01 in Student's t-test.

c. Chlorophyll content

Chlorophyll contents of only *grf3* have distinct difference after 5 days after white light. The possible reason can be that the selected targets are not involved in the pathway of chlorophyll biosynthesis or that there are so many factors controling the biosynthesis of chlorophyll. We conclude that GRF3 (Growth-Regulating Factor 3) could positively influence the content of chlorophyll (Figure 26).

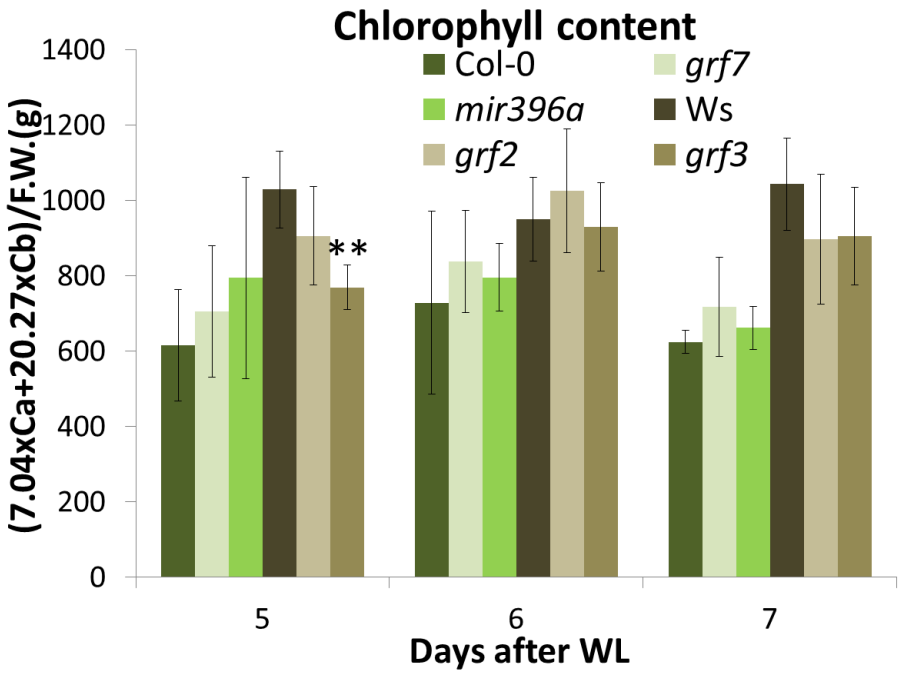


Figure 26. *grf3* has less chlorophyll content after 5 days in WL (White Light). The error bars indicate mean ± STD from 5-11 biological repeats. "*" indicates $P<0.05$; "**" indicates $P<0.01$ in Student's t-test.

d. Cotyledon area

MIR396A and *grf7* have different size of cotyledons, and both of theirs are smaller than wild type (Col-0 and Ws). Not only do miR396a and GRF3 affect the final size of cotyledons, but they also influence the development rate of cotyledon, which are not very apparent though according to the results. We conclude that miR396a and GRF2 (Growth-Regulating Factor 2) could positively influence the cotyledon development (Figure 27).

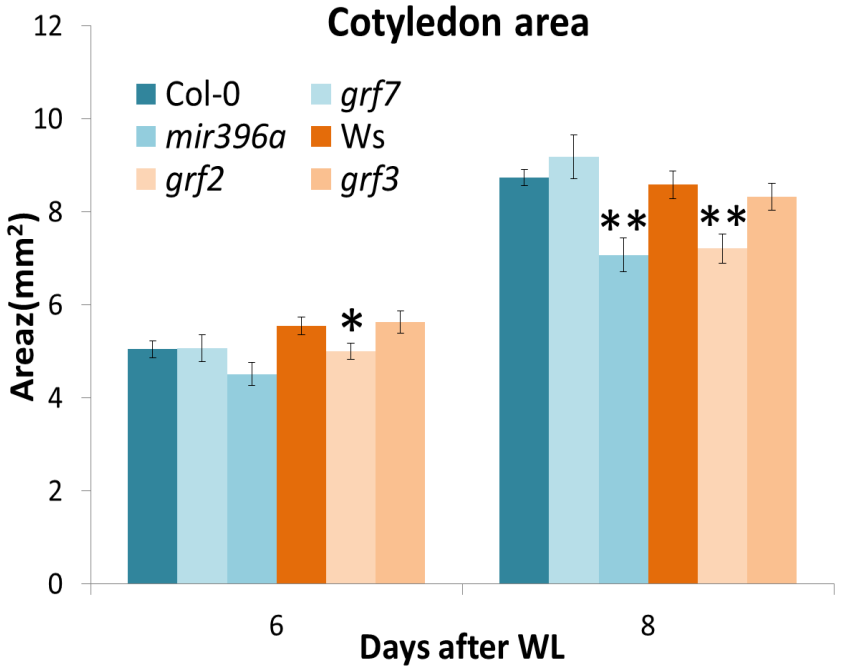
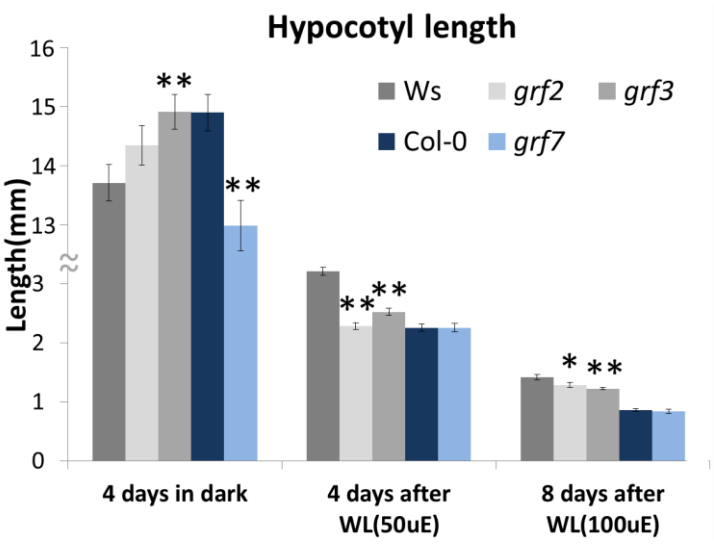


Figure 27. *mir396a* and *grf2* have smaller fully open cotyledons and the cotyledons of *grf2* also develop more slowly in WL (White Light). The result is one of the 3 biological repeats. The error bars indicate mean ±SE. "*" indicates P<0.05; "**" indicates P<0.01 in Student's t-test.

e. Hypocotyl length

grf3 and *grf7* have different length of hypocotyls in dark, and the former has longer hypocotyls and the latter has much shorter hypocotyls than wild type (Col-0 and Ws). It seems that GRF7 plays a crucial role increasing hypocotyl elongation. Not only do GRF2 and miR396a promote hypocotyl elongation in white light, but GRF3 also influences it. However, not like in dark, *grf3* shows shorter hypocotyls in white light. We conclude that miR396a, GRF2 (Growth-Regulating Factor 2) and GRF3 (Growth-Regulating Factor 3) could positively influence the hypocotyl elongation in white light and that GRF2 (Growth-Regulating Factor 2) and GRF7 (Growth-Regulating Factor 7) play opposite roles in the process (Figure 28).



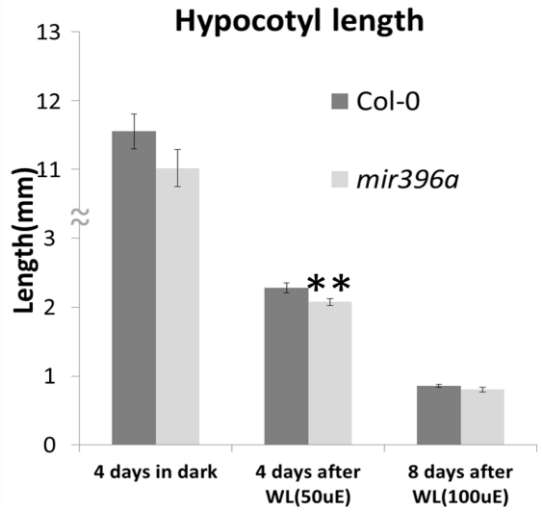
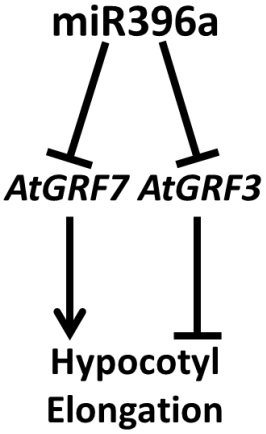


Figure 28. The hypocotyls of *grf2*, *grf3* and *mir396a* develop more slowly in WL (White Light) while *grf2* and *grf3* has shorter fully-elongated hypocotyls in WL; *grf3* have longer hypocotyls while *grf7* has shorter hypocotyls after 4 days in dark. The result is one of the 2-3 biological repeats. The error bars indicate mean ±SE. "*" indicates P<0.05; "**" indicates P<0.01 in Student's t-test.

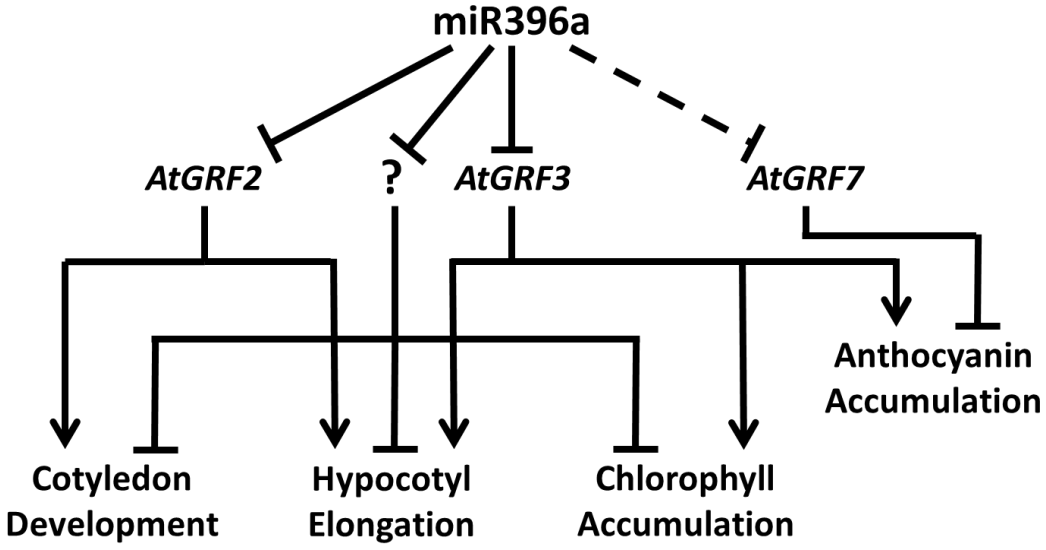
f. Proposed model

Dark



Due to opposite functions of the two miR396 targets involved in hypocotyl elongation in dark, that *mir396a* does not show obviously different hypocotyls in dark in our experiments is reasonable.

Light



The lower part of the schema displays the brief results of our tests into the targets. And the upper part is what miR396a supposed to do to the targets. By comparing and combining the results, we can get this schema. miR396a may repress anthocyanin accumulation by decreasing *AtGRF3*, while the pathway through *AtGRF7* may not be a considerable regulation track. As to the other morphological phenotypes, AtGRF2 and AtGRF3 have individual functions, but both are connected to promoting growth. However, *mir396a* shows the same phenotypes with the target mutants, so there may be other miR396-target genes involved in the process.

2. miR858

a. The mutants used in observing the phenotypes (Figure 29)

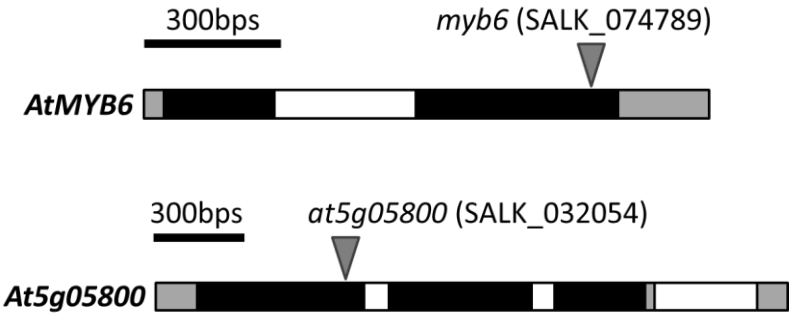


Figure 29. Schematic gene structures showing T-DNA insertion sites marked with arrowheads. Black, gray and white bars indicate exons, non-translated regions and introns respectively.

b. Anthocyanin content

Anthocyanin content in *at5g05800* is higher than wild type (Col-0) after 5 to 6 days after white light, which is not like the cases in the targets of miR396. The mutants of miR396 targets show the biggest difference in 4 days after white light while the difference of *at5g05800* emerge quite later. We conclude that At5g05800 limits the anthocyanin content in white light but may not have direct relationship with light (Figure 30).

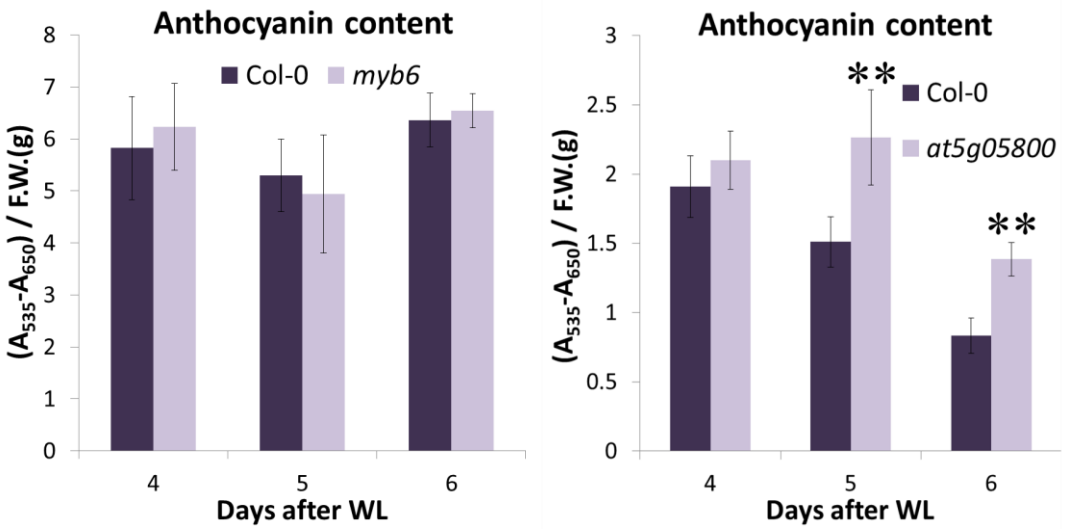


Figure 30. *at5g05800* has more anthocyanin content after 5 and 6 days in WL (White Light). The error bars indicate mean ± STD from 4-6 biological repeats. Media used are with 1%sucrose to increase anthocyanin. "*" indicates $P<0.05$; "**" indicates $P<0.01$ in Student's t-test.

c. Chloropphyll content

Just like the situation in *grf2*, *myb6* has the difference from the wild type (Col-0) in 5 days after white light. Nonetheless, *myb6* has higher chlorophyll content. We conclude that MYB6 (MYB DNA-binding protein 6) limits the chlorophyll biosynthesis (Figure 31).

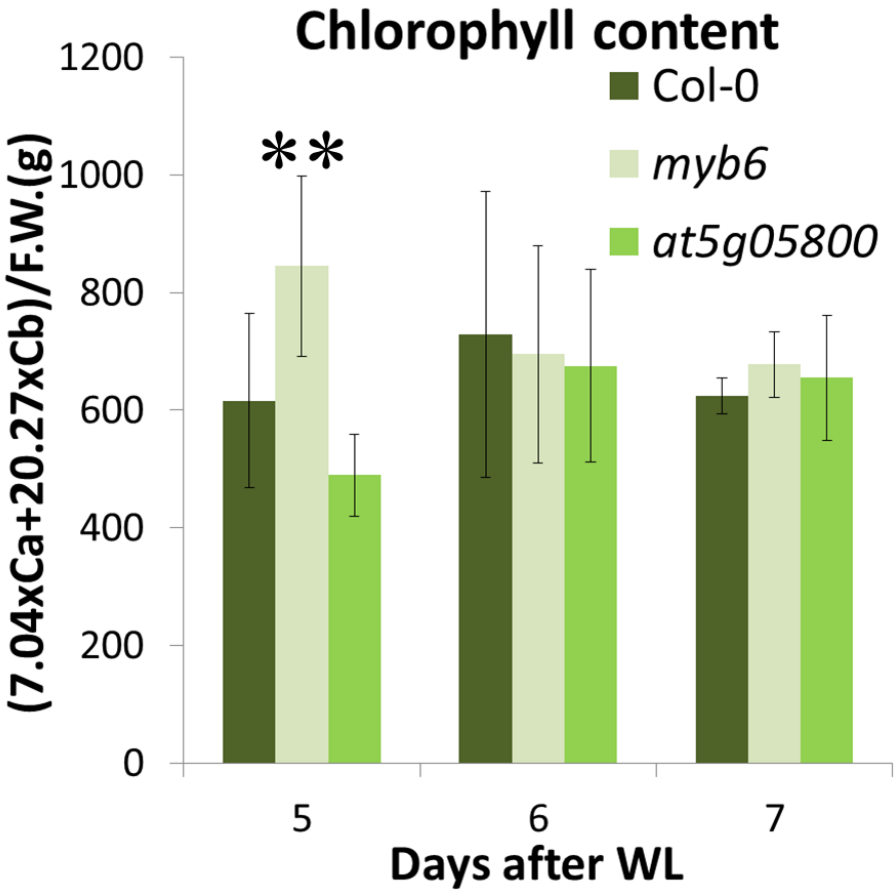


Figure 31. *myb6* has more chlorophyll content after 5 days in WL(White Light). The error bars indicate mean ± STD from 5-11 biological repeats. "*" indicates $P<0.05$; "**" indicates $P<0.01$ in Student's t-test.

d. Cotyledoon area

Unlike *grf2*, *myb6* does not show defectives in the development rate of cotyledons. On the other way, MYB6 is more like a cotyledon-size restricter than a promoter of cotyledon development. We conclude that MYB6 (MYB DNA-binding protein 6) could be a restricter in cotyledon development in white light (Figure 32).

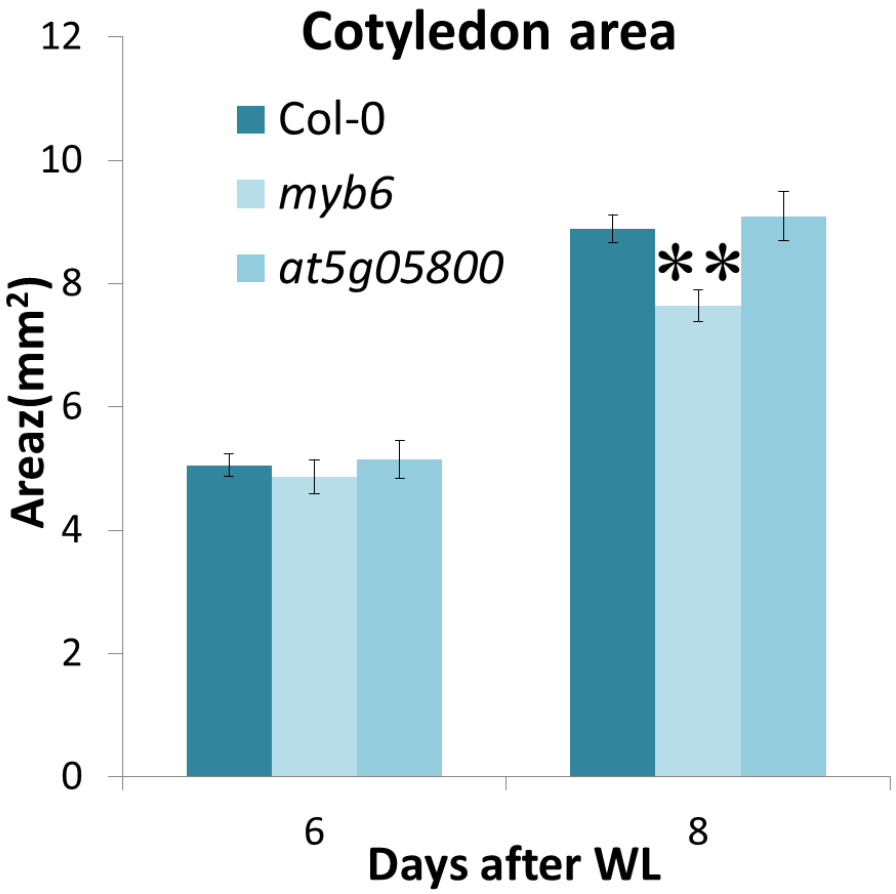


Figure 32. *myb6* has smaller fully open cotyledons in WL (White Light). The result is one of the 3 biological repeats. The error bars indicate mean ±SE. "*" indicates P<0.05; "**" indicates P<0.01 in Student's t-test.

e. Hypocotyl elongation

Like *grf2* and *grf3*, *myb6* has shorter hypocotyls than wild type (Col-0), whereas *myb6* seems not connected to the elongation rate of hypocotyls in white light. In contrast, *at5g05800* only shows difference in dark. We conclude that MYB6 (MYB DNA-binding protein 6) could be a restricter in hypocotyl elongation in white light and that At5g05800 is a promoter of hypocotyl elongation in dark.

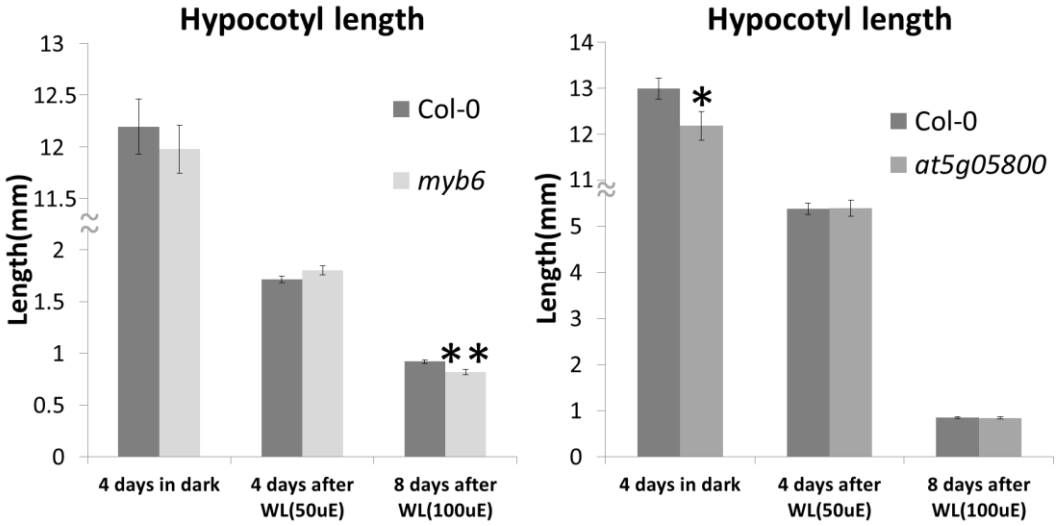
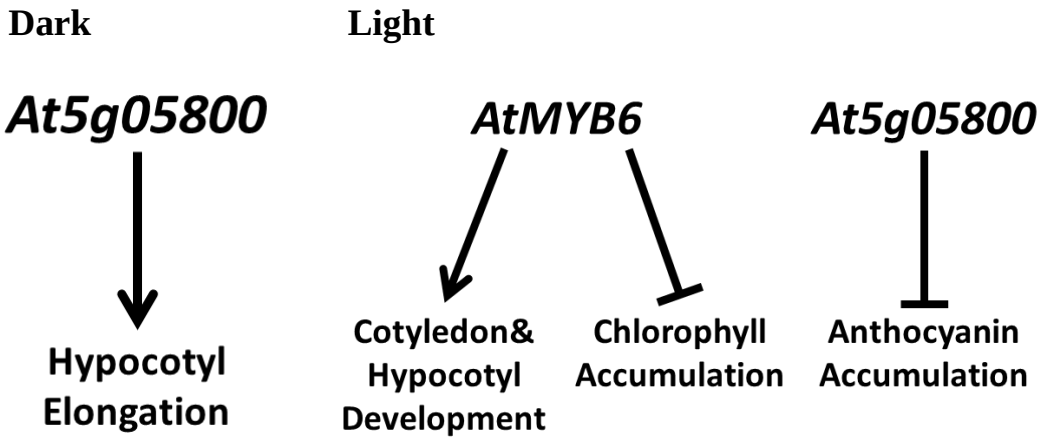


Figure 33. *at5g05800* has shorter hypocotyl after 4 days in dark; *myb6* has shorter fully-elongated hypocotyl in WL (White Light). The result is one of the 2-3 biological repeats. The error bars indicate mean ±SE. "*" indicates $P<0.05$; "**" indicates $P<0.01$ in Student's t-test.

f. Proposed model



Unlike *AtGRF2* or *AtGRF3*, *AtMYB6* focuses on cell enlargement and elongation in light. The role of *At5g05800*, as a restricter of anthocyanin accumulation, may be more likely to be development-induced because the difference comes up quite late after white light exposure.

Discussion

In our research, we select *Arabidopsis thaliana* as our object of study. The reason is that it is the model plant in botany. On the other hand, the previous research and databases is rich. Here we choose Col-0 and Ws as our wild types.

The targets of miR396 include the *GRF* family (*GRF*1, 2, 3, 4, 7, 8, 9), which is involved in cotyledon opening (Dongmei Liu, 2009) . While the majority of the predicted targets of miR858 are the members of MYB family, acting as transcription factor involved in synthesis of lignin (*MYB63*) (Jae-Heung Koand, 2009) and accumulation of anthocyanin (*MYBL2*) (Christian Dubos, 2008), etc. We hypothesize the products of these genes may be specific factors in photomorphogenesis. The imformation of the predicted targets is useful. When we actually observe the phenotypes, we can use these to explain what we infer. Those targets we exclude do not have obvious anti-correlation with the miRNAs; however, they may be truly degraded.

Although NGS is a nice way to do wild-ranged prediction, it somehow is not accurate. In order to prove the results of NGS, we perform northern blotting to confirm the expression patterns of selected miRNAs, and Real-Time PCR to check the targets.

In northern blotting, if there is no image on the right sites of phosphoimaging plate or brightness is too low, we will exclude it after exposing the membrane to

phosphoimaging plate for 3~5 days. Our choosing is based on how distinct the change pattern is and the comparison between northern blotting and NGS data.

Otherwise, northern blotting can not differentiate miRNAs in the same family due to their similar sequence, and neither can Real-Time PCR differentiate the various mature sequence due to the primers used in are similar. So when discussing the relationship between the miRNAs and the targets, we have to take it into consideration.

After finishing the selection of miRNA-target pairs, we get miRNA396a/b, miRNA858a/b and conduct Real-Time PCR for their predicted targets. Real-Time PCR is conducted over the targets of which Pearson correlation coefficient with the miRNAs less than -0.5. For further verification, 5'RACE (5' Rapid Amplification of cDNA ends) is a choice. By checking the cleavage events, there is one more evidence proving the relationship between the miRNA-target pairs.

Although we have performed experiments to verify transcriptional regulation between miRNAs and targets, we would like to understand the physiological functions of miRNAs in photomorphogenesis. To resolve the doubt, we get the mutants of the miRNAs and targets from Salk Institute to observe the phenotypes of the plants in order to prove whether the miRNAs really affect photomorphogenesis and how they affect it. In this respect, we emphasize on figures of the seedlings after several days of white light exposure, for example, cotyledon opening and hypocotyls shortening. We first select homozygousities since the heterozygous mutants can not display clear phenotypes due to a normal gene still remaining. The seeds they offered are not all homozygous according to our experience, and that is why we conduct genotyping.

But *MIR858* single mutants and *At1g80060* mutants are not available, and there might not be obvious phenotypes due to functional redundancy within miRNA families, so we need to build target mimicry lines and observe the morphology of the plants. Also, the targets mimicry and miRNA mutants lines are the opposite in-plant situation to the miRNA overexpression lines. By comparing to the phenotypes of these lines, we can make the conclusion about the roles miRNAs play in photomorphogenesis.

In the current steps, we have investigate all homozygous T-DNA lines of mutants we ordered and selected T3 of targets mimicry lines. The future work will mainly be focused on investigating the phenotypes of these transgenic lines,and we hope to discover the function of miRNA-target pairs that are involved in *Arabidopsis* photomorphogenic growth.

Conclusions

A. The expression of *MIR396* and *MIR858* is regulated by light in de-etiolating *Arabidopsis* seedlings.

B. miR396 may repress *AtGRF2, AtGRF3, AtGRF7* and *At1g80060* in de-etiolating *Arabidopsis* seedlings.

C. miR858 may repress *AtMYB6* and *At5g05800* in de-etiolating *Arabidopsis* seedlings.

D. *AtGRF3* increases anthocyanin content while *AtGRF7* and *At5g05800* repress it.

E. *AtGRF3* increases chlorophyll content while *AtMYB6* represses it .

F. *AtGRF2* and AtMYB6 both promote cotyledon development.

G. *AtGRF2, AtGRF3* and *AtMYB6* promote hypocotyl elongation in white light.

H. *AtGRF7* promotes hypocotyl elongation while *AtGRF3* and *At5g05800* limit it.

Future work

A. Finish observing the phenotypes of the target mimicry / miRNA overexpression lines.

B. Conduct northern blotting over the miRNAs and Real-Time PCR over the corresponding targets in the target mimicry / miRNA overexpression lines

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Appendix

Isolate total RNA

1. Get 1000~2000 seedlings (a medium) grinded under each process
2. Add 1 c.c. Plant RNA Purification Reagent (Life technologies Cat# 12322-012)
3. Wait for 10 minutes at room temperature
4. Centrifuge for 10 minutes at 13000rpm
5. Add 200μL $NaCl_{(aq)}$(5M) to new eppendorfs
6. Take supernatant liquid into eppendorfs
7. Add 600μL the solution composed of Chloroform and Isoamyl alcohol (24:1)
8. Vortex
9. Centrifuge for 15 minutes at 13000rpm
10. Take supernatant liquid (900μL) into eppendorfs
11. Add 900μL Isopropyl alcohol
12. Vortex
13. Wait for 10 minutes at room temperature
14. Centrifuge for 20 minutes at 13000rpm
15. Pour off supernatant liquid
16. Add 1c.c. ethanol (95%)
17. Centrifuge for 5 minutes at 13000rpm
18. Pour off ethanol
19. Dry pellet by SpeedVac for 2 to 3 minutes.

Isolate total DNA in order to do Genotyping, make templates or conduct Southern blotting

A. Slice a leaf from adult *Arabidopsis thaliana* and put one into an eppendorf

B. Isolation

1. Grind leaf with shaking little steelball for 20 seconds at 1200 rpm
2. Repeat step one again
3. Add 2X CTAB (heated to 65℃) buffer 0.5 c.c. into each eppendorf

2X CTAB buffer	Tris-HCl (pH 8.0)	100mM
	NaCl	1.4M
	EDTA	20mM
	CTAB	2%
	PVP40	2%
	β-mercaptoethanol	0.2%

4. Get the steel balls out of the eppendorfs
5. Add 500μL the solution composed of Chloroform and Isoamyl alcohol (24:1) and shake it by hands
6. Centrifuge for 15 minutes at 13000 rpm
7. Get supernatant liquid 400μL
8. Add 400μL Isopropyl alcohol
9. Shake it by hands and settle for overnight at 4℃

Purification of DNA from gel/ PCR product

(Using Gene-Spin^{TM}-V^{2}, 1-4-3 DNA Extration Kit Cat# RT-DNA143V$_2$)

1. Put the gel sliced containing a band into a 1 c.c. eppendorf
   (Purification of PCR product: Add 70μL ddH_2O to PCR product, and move it into a 1 c.c. Eppendorf)
2. Add binding Buffer till the volume is about 1 c.c.
3. Wait for 10 minutes at 65℃
4. Get the liquid onto the columns
5. Centrifuge for 1 minute at 13000rpm
6. Get the flow through onto the columns again
7. Change a new set of eppendorfs
8. Add 700μL Wash Buffer
9. Centrifuge for 1 minute at 13000rpm
10. Pour off the flow through
11. Centrifuge for 2 minutes at 13000rpm
12. Prepare a new set of eppendorfs and put the columns with purified plasmid on eppendorfs
13. Add 20-40μL ddH_2O
14. Centrifuge for 2 minutes
15. Detect the concentration of DNA by Nanodrop

Northern blotting for small RNA

A. Transfer RNA to membrane

1. Add 100μL water to total RNA samples
2. Measure the concentration of RNA by Nanodrop
3. Get volume including 20μg and add water to 100μL
4. Dry it and make its volume decrease to 15μL
5. Add 15μL 2X TBE-UREA Sample Buffer
6. Heat sample at 95℃ for 10 minutes and stick into ice immediately
7. Load 30μL sample into each well, and Marker1 is Biolabs #N2102S as well as Marker2 is Biolabs #B0362S

Marker1	Maker2	0hr	1hr	3hr	6hr	12hr	24hr

8. Run gel at 120 volt for 150 minutes
   (0.5X TBE buffer, TBE-Urea Gel 15% / 10 wells gel)
9. Put each gel into 50ml 0.5X TBE buffer.
10. Add 2.5μL ABI SYBR gold to each gel (SYBR® Gold Nucleic Acid Gel Stain. Cat# S-11494)
11. Cover it with aluminium foil and shake it for 30 minutes at 60 rpm
12. Take photos (Exposure time: 0.5-2.0 seconds)
13. Transfer for 45 minutes at 400mA
    (Membranes are from GE Healthcare)
14. UV-crosslink for about 2 minutes (About 2400 J)

B. Probe labeling

1. Label buffer (Each membrane uses 20μL)

Label buffer	ddH_2O	10 μL
	10X T4-PNK buffer (Biolabs, Cat#B0201S)	2 μL
	Primer	2 μL
	T4-PNK (Biolabs, Cat# M0201)	1 μL
	γ-32P-ATP	5 μL
	Total	20 μL

(ATP, [γ-32P]-10Ci/mmol 2mCi/ml, 250 μCi, PerkinElmer, Cat#BLU002250UC)

PNK buffer	Tris-HCl	70mM
	$MgCl_2$	10mM
	DTT	5mM
	pH:7.6 (At 25℃)	

2. Wait for 1 hour at 37℃
3. Centrifuge empty column (illustra Microspin G-25 Columns) for 1 minute at 3000 rpm
4. Change eppendorfs under the columns
5. Add 30μL ddH2O to eppendorfs containing probe
6. Add the probe onto the columns
7. Centrifuge for 2 minutes at 3000 rpm
8. Put one membrane into each plastic tube containing UltraHyb-Oligo buffer (Ambion, Life Cat#AM8663) (5 ml hybridization buffer) and spin it for 2 hours at 12 rpm at 37 ℃
9. Add the isolated probe liquid to plastic tubes and hybridize for overnight at 12 rpm at 37 ℃

C. Prepare for Phosphoimaging Plate

1. Heat Wash I buffer to 37℃
   (Wash I buffer: 2X SSC, 0.1% SDS)(
2. Spin one membrane in 200ml Wash I buffer at 60 rpm for 10 minutes at 37℃
3. Repeat step 2 again
4. Spin one membrane in 200ml Wash II buffer at 60 rpm for 2 minutes at 27℃
   (Wash II buffer: 1X SSC, 0.1% SDS)
5. Dry membranes on tissue papers
6. Expose the membrane to phosphoimaging plate for 3~5 days.
   (SSC 20X buffer, Bioman, Cat# SSC205000)
   (SDS 10% solution, Bioman, Cat# SDS101000)

D. After Phosphoimaging Plate

1. Heat Tris-buffer to 90℃
   (Tris-buffer: 0.2 % SDS, 10mM Tris, pH7.5)
2. Soak membranes in Tris-buffer for 1 minute at 90℃

Reverse transcription (Using SuperScript® II Reverse Transcriptase Cat#18064-014)

1. Samples are: Col-0

(Dark 4 days then White light 0, 1, 3, 6, 12, 24 hrs)(three biological repeats)

2. Isolate RNA

3. DNase treatment

RNA	8 μL (total 2 μg RNA)
DNase buffer	1 μL
DNase	1 μL
Total	10 μL

DNase buffer	Tris-HCl	10mM
	$MgCl_2$	2.5mM
	$CaCl_2$	0.5mM
	pH7.6 at 25℃	
	Storage: -20℃	

4. Wait for 30 minutes at 37℃

5. Primer binding

DNase treated RNA	10 μL	
Oligo-dT (3μg/μL)	0.25 μL	13X master mix
dNTP (10mM) (GM Cat#GM007)	1 μL	
DEPC-H_2O	0.75 μL	
Total	12 μL	

6. Wait for 10 minutes at 65℃ and immediately move samples to 4℃

7. Reverse transcript

Primer bound RNA	12 μL	
DEPC- H_2O	0.5 μL	13X master mix
5X FS buffer	4 μL	
0.1M DTT	2 μL	
rRNA sin	1 μL	
SSII-RT	0.5 μL	
Total	20 μL	

8. Wait for 1 hour at 42℃

9. Wait for 15 minutes at 70℃

10. Dilute to 0.05ng/μL (20ng/400μL)

(Reverse transcript rate is about (20ng cDNA/ 2μg RNA))

Real-Time PCR

1.

a. For observing the quantity of the targets at each time point in photomorphogenesis: Samples are from each light condition (Dark 4 days then White light 0, 1, 3, 6, 12, 24 hrs)

b. For checking the quantity of the targets in mutants: Samples are from the mutant seedling after white light exposure 0hr and 1hr

2. PCR materials

	1X (μL)	19.5(μL)
cDNA(0.05ng/μL)	5	-
FW primer (5μM)	1	19.5
RV primer (5μM)	1	19.5
ddH_2O	3	58.5
Power SYBR® Green PCR Master Mix (Cat#4367659)	10	195
Total	20	

3. Arrangement

a. For observing the quantity of the targets at each time point in photomorphogenesis:

1.	Target gene	6 (time spots)X3 (Technical repeats)+Control=19
2.	UBQ10	6 (time spots)X3 (Technical repeats)+Control=19

(Control: Replace cDNA with water)

b. For checking the quantity of the targets in mutants:

1.	cDNA from the mutants	2 (time spots)X3 (Technical repeats)+Control=7
2.	cDNA from the wild type	2 (time spots)X3 (Technical repeats)+Control=7

(Control: Replace cDNA with water)

Genotyping for homozygositycheck

1. PCR materials

	1X (μL)	7.5 (μL)
Template (From purified total DNA)	1	-
R. Buffer (10X) with 15mM $MgCl_2$ (Cat#JMR-420)	2	15
FW primer (10μM)	1	7.5
RV primer (10μM)	1	7.5
dNTP (10mM) (GM Cat#GM007)	0.4	3
ddH2O	14.6	109.5
Super Therm Taq DNA polymerase (Medox-Bio® Cat# MX-0647)	0.04	0.3
Total	20.04	

PCR condition

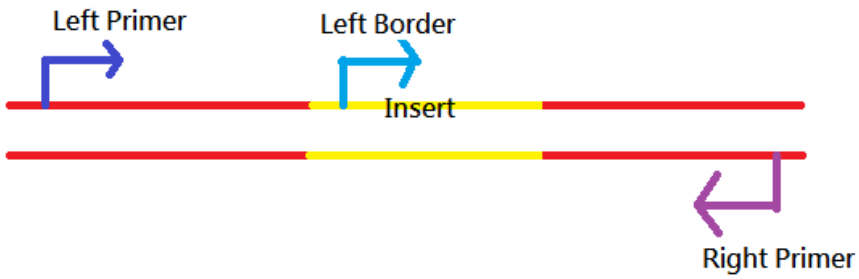
95℃	5 minutes	
95℃	30 seconds	35 cycles
55℃	30 seconds	
72℃	1.5 minutes	
72℃	5 minutes	
4℃	Pause	

2. Mix two products from the same plant but using different primers into one

	FW primer	RV primer
PCR product 1	Left Primer	Right Border
PCR product 2	Left Primer	Right Primer

(LBb1.3 is used as Left Border)

LBb1.3	ATTTTGCCGATTTCGGAAC



(Diagram explaining Left Primer, Right Primer and Left Border)

3. Add 6X dye 4 μL to each eppendorf

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

4. Eletrophoresis (1% TAE-Agarose gel, 100 volt, 20 minutes) (Each sample is loaded 10μL) (Marker: 1 Kb Plus DNA)

Marker (1 Kb Plus DNA)	Col-0	SALK mutant 1	SALK mutant 2	SALK mutant 3	SALK mutant 4	SALK mutant 5	SALK mutant 6

Northern blotting for mRNA

A. Synthesis of DIG-labeled probe by PCR

1. PCR materials

	DIG-labeled (μL)	Non-labeled (μL)
cDNA Template (5ng/μL)	2	2
Super Therm Taq DNA polymerase (Medox-Bio® Cat# MX-0647)	0,1	0.1
FW Primer (10μM)	2.5	2.5
RV Primer (10μM)	2.5	2.5
Digoxigenin-11-dUTP, Roche, Cat#11093088910	1	-
dNTP (10mM) (GM Cat#GM007)	1	1
R. Buffer (10X) with 15mM $MgCl_2$ (Cat#JMR-420)	5	5
ddH_2O	35.9	36.9
Total	50	50

PCR condition

95℃	5 minutes	
95℃	30 seconds	35 cycles
55℃	30 seconds	
72℃	1.5 minutes	
72℃	7 minutes	
4℃	Pause	

2. Get 2μL to conduct (1% TAE-Agarose gel, 120 volt, 15 minutes) (Checking the quantity of probe)
3. Purification of PCR product

B. Electrophoresis of mRNA sample (Total RNA)

1. Prepare formaldehyde gel

Agarose	0.6g
20X MOPS (J.T.Baker® , Cat#4004-05)	2.5mL
DEPC-H_2O	47.5mL
EtBr (SIGMA-ALDRICH, Cat#E1510) (Add it at last)	2μL
Total	50mL (A piece of gel)

20X MOPS (pH is adjusted to 7.0 with NaOH)	
MOPS	83.73g
NaOAc	8.2g
EDTA, O.5M, pH 8,0	40 mL
Add H_20 to 1 L	

2. Let the gel solidify
3. Place the gel into 1X MOPS running buffer

1X MOPS running buffer	
20X MOPS	25mL
H_2O	475 mL
Total	500 mL

3. Mix RNA sample (20μg/20μL) with 5X RNA loading buffer (5μL)

5X RNA loading buffer (10mL)	
EDTA, O.5M, pH 8,0	80μL
Formaldehyde (25%)	720μL
Glycerol (100%)	2mL
Formamide	3.084mL
20X MOPS	2 mL
Add DEPC-H2O to 10mL and store it at 4℃, avoiding light	

4. Heat the samples at 65℃ for 10 minutes, and place them onto the ice quickly
5. Wait for 5 minutes
6. Run the gel for at 80 vote 1.5hrs and check the result and take pictures

C. Transfer by vacuum blotting (VacuGene XL Vacuum blotting System)

1. Keep the gels and membranes wet with 10X SSC
2. Keep the pressure up to 100 mbar
3. Transfer for 1hr
4. UV-crosslink for about 2 minutes (About 2400 J)

D. Hybridization

1. Pre-hybridize for 2hrs at 12rpm at 60℃
   (FastHyb-Hybridization Solution, BioChain, Cat#L1031250)
2. Heat the probe solution at 95℃ for 5 minutes and place it onto the ice quickly
3. Wait for 5 minutes
4. Add the probe solution in the plastic tube to the hybridization buffer and spin it at 12 rpm at 60℃ overnight

E. Wash

1. Add Wash I buffer 100mL to a plastic box
2. Move the membrane into the buffer and spin it at 60rpm for 5 minutes at RT
3. Pour off the buffer
4. Add Wash I buffer 100mL to the plastic box and spin it at 60rpm for 5 minutes at RT again
5. Pour off the buffer
6. Add Wash II buffer 100mL to the plastic box (Heated to 60℃)
7. Spin it at 60rpm for 5minutes at 60℃
8. Pour off the buffer
9. Repeat step 6-8 again

Wash buffer I		Wash buffer II	
20X SSC	10mL	20X SSC	5mL
10%SDS	10mL	10%SDS	10mL
Add H_2O to 1L		Add H_2O to 1L	

(SSC 20X buffer, Bioman, Cat# SSC205000)
(SDS 10% solution, Bioman, Cat# SDS101000)

F. Preparation for detection

1. Dilute 10X Maleic acid buffer into 1X Maleic acid buffer

10X Maleic acid buffer (pH is adjusted to 7.5 with NaOH)	
Maleic acid	116.08g
NaCl	58.44g
Add H_20 to 1 L	

2. Mix 45mL 1X Maleic acid buffer and 5mL 10X Blocking buffer (5g Blocking Reagent, Roche, Cat#11096176001) to make 1X Blocking buffer

3. Add Tween 20 wash buffer 100mL to the plastic box with the membrane in

Tween 20 wash buffer	
Tween 20 (100%)	0.3%
In 1X Maleic acid buffer	

(TWEEN® 20, SIGMA, Cat#P9416 SIGMA)

4. Spin it at 60rpm for 5minutes at RT
5. Pour off the buffer
6. Add 15mL 1X Blocking buffer
7. Spin it at 60rpm for at least 30 minutes but no more than 3hrs at RT
8. Pour off the buffer
9. Add 20mL 1X Blocking buffer containing 2μL anti-DIG-AP
   (Anti-Digoxigenin-AP, Fab fragments, Cat#11093274910)
10. Spin it at 60rpm for at least 30 minutes but no more than 3hrs at RT
11. Pour off the buffer
12. Add 100-200mL 1X Maleic acid buffer to the plastic box
13. Spin it at 60rpm for 15 minutes at RT
14. Pour off the buffer
15. Repeat step 12-14 again
16. Add 10mL detection buffer to the plastic box

Detection buffer (1L)	
1M Tris-HCl (pH 9.5)	100mL
5M NaCl	20mL
H_2O	880mL

17. Spin it at 60rpm for 5 minutes at RT
18. Pour off the buffer
19. Dilute 20μL CDP (CDP-Star® , SIGMA, Cat#C0712) into 2mL detection buffer
20. Add the buffer onto the membrane and put it into a plastic bag

G. Detection

1. Expose the film to the membrane for 30 minutes to several hours
   (Amersham Hyperfilm MP, GE Healthcare Life Sciences, Cat# 28-9068-42)

PCR and electrophoresis to get *IPS1*

1. Touch-up PCR

PCR materials

Template (Purified total DNA)	5μL
5X PCR buffer (BIO-RAD 5X iProof HF Buffer#172-5391)	10μL
10μM FW primer(With *Sal*I enzyme site)	2.5μL
10μM RV primer (With *Sac*I enzyme site)	2.5μL
10mM dNTP (GM Cat#GM007)	1μL
iProof High-Fidelity DNA Polymerase (BIO-RAD Cat#172-5301)	0.5μL
ddH_2O	28.5μL
Total	50μL

PCR condition

95℃	5 minutes	
95℃	30seconds	5 cycles
58℃	30seconds	
72℃	30 seconds	
95℃	30 seconds	35 cycles
68℃	30 seconds	
72℃	1 minute	
72℃	5 minutes	
4℃	Pause	

2. Enzyme site (For cloning to Binary vector(*35S::*pCambia 1390))

Primer	Enzyme cut site	Sequence of cleavage site
FW primer	*Sal*I	GTCGAC
RV primer	*Sac*I	GAGCTC

3. Add 6X dye10μL to each eppendorf

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

4. Eletrophoresis (1% TAE-Agarose gel, 130 volt, 15 minutes)
   (First, load 10μL. If product exists, second load 50μL)
5. Purification DNA from gel
6. A-Tailing of purified DNA
7. Ligation to T-A vector (pGEM T-easy)
8. Transform to *E. coli* and stock *E.coli*

Make abundant inserts of T-A vector(pGEM T-easy) in overexpressing miRNA

1. PCR materials

Template (Purified total DNA)	1μL
5X PCR buffer (BIO-RAD 5X iProof HF Buffer#172-5391)	10μL
10μM FW primer (With *Sal*I enzyme site)	2.5μL
10μM RV primer (With *Sac*I enzyme site)	2.5μL
10mM dNTP (GM Cat#GM007)	1μL
iProof High-Fidelity DNA Polymerase (BIO-RAD Cat#172-5301)	0.5μL
ddH_2O	28.5μL
Total	50μL

PCR condition

MIR396B			*MIR858A*		
98℃	30 seconds		98℃	30 seconds	
98℃	10 seconds	35 cycles	98℃	10 seconds	35 cycles
50℃	30 seconds		45℃	30 seconds	
72℃	2 minutes		72℃	1 minute	
72℃	5 minutes		72℃	5 minutes	
4℃	Pause		4℃	Pause	

2. Enzyme site (For cloning to Binary vector(*35S::*pCambia 1390))

Primer	Enzyme cut site	Sequence of cleavage site
FW primer	S*al*I	GTCGAC
RV primer	S*ac*I	GAGCTC

3. Add 6X dye10μL to each eppendorf

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

4. Eletrophoresis

(1% TAE-Agarose gel, 124 volt, 25minutes) (Marker: 1 Kb Plus DNA)

5. Purification DNA from gel

PCR product A tailing and Eletrophoresis to confirm the size

1. Add 0.1μL Super Therm Taq DNA polymerase(Medox-Bio® Cat# MX-0647) to 50μL PCR product
2. Wait for 10 minutes at 72℃
3. Add 6X dye 10 μL to each eppendorf

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

4. Each sample is separated to two part using two well
5. Eletrophoresis
   (1% TAE-Agarose gel, 120 volt, 20 minutes) (Marker: 1 Kb Plus DNA)

A- Tailing of purified DNA
(used in cloning MIR396, MIR858 and *IPS1* to pGEM T-easy)

1. Condition

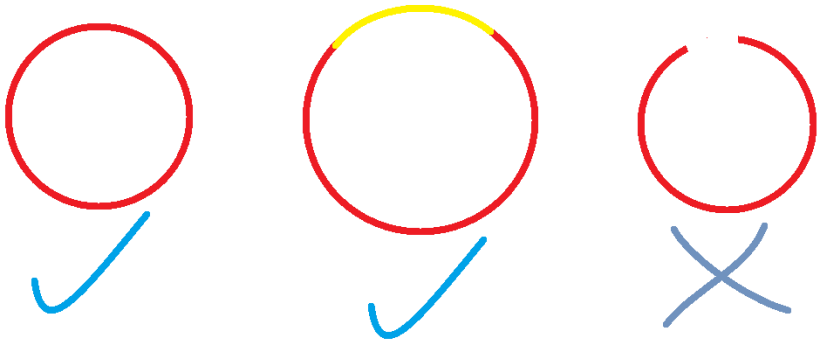
	1X (μL)	3.5X (μL)
Sample (Purified MIR396/ MIR858)	15	-
R. Buffer (10X) with 15mM $MgCl_2$ (Cat#JMR-420)	2	7
dNTP (10mM) (GM Cat#GM007)	0.4	1.4
Super Therm Taq DNA polymerase(Medox-Bio® Cat# MX-0647)	0.04	0.14
ddH2O	2.6	9.1

2. Wait for 15 minutes at 72℃

Colony PCR

A. Preparation before colony PCR

1. Cultivate *E. coli* on Ampicillin medium at 37℃ while *Agro* on Gentamicin medium at 28℃ for 1 hour after transformation
2. Select 23 colonies and cultivate *E. coli* at 37℃ while *Agro* at 28℃ with slight shake for 16 hours on another Bacteria medium with Ampicillin for *E. coli* or Gentamicin for *Agro*



(Red part is vector and yellow one is insert. Bacteria can survive both in the first and the second condition, while the last one will be degraded in bacteria. So, we can compare the numbers of colonies with insert or not; then, we will know the success rate of insertion)

B. PCR

1. 23 colonies random-selected + medium (control) = 24

	1X (μL)	25X (μL)
Template	-	-
R. Buffer (10X) with 15mM $MgCl_2$ (Cat#JMR-420)	2	50
10μM FW primer	1	25
10μM RV primer	1	25
dNTP (10mM) (GM Cat#GM007)	0.4	10
Super Therm Taq DNA polymerase(Medox-Bio® Cat# MX-0647)	0.04	1
ddH_2O	15.6	390
Total	20	500

PCR condition

35S::MIR396B/pCambia 1390 & *35S::MIR396B*/pCambia 1390			The others		
95℃	5 minutes		95℃	5 minutes	
95℃	30 seconds	35 cycles	95℃	30 seconds	35 cycles
55℃	30 seconds		55℃	30 seconds	
72℃	2 minutes		72℃	1 minute	
72℃	5 minutes		72℃	5 minutes	
4℃	Pause		4℃	Pause	

2. Add 6X dye 4 μL to each eppendorf

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

C. Eletrophoresis

(1% TAE-Agarose gel, 100 volt, 15 minutes) (Each sample is loaded 10μL)

(Marker: 1 Kb Plus DNA)

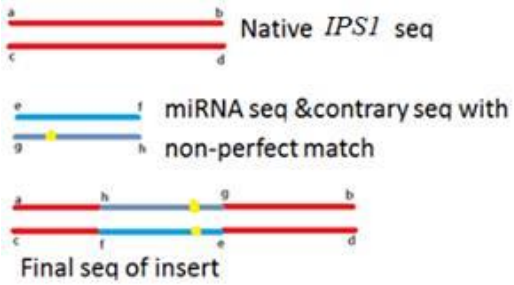
Plasmid purification from *E. coli*

(Using Molecular Biology Tools GM Mini Plasmid kit Cat# DPolm-50)

1. Centrifuge 1.5 c.c. LB including *E. coli* at 13000rpm for 1 minute
2. Pour off the supertanant
3. Add Buffer 1 200μL and vortex
4. Add Buffer 2 200μL and shake it by hands
5. Add Buffer 3 200μL and shake it by hands
6. Centrifuge for 10 minutes at 13000 rpm
7. Get supernatant liquid 600μL and add it onto the column
8. Centrifuge for 1 minute at 13000 rpm and pour off the flow through
9. Add Wash Buffer 600μL
10. Centrifuge for 1 minute at 13000 rpm and pour off the flow through
11. Add Wash Buffer 300μL
12. Centrifuge for 1 minute at 13000 rpm and pour off the flow through
13. Centrifuge for 2 minutes
14. Prepare a new set of eppendorfs and put the columns with purified plasmid on them on eppendorfs
15. Add ddH_2O (pGEM T-easy : 40~50μL ; *35S::*pCambia 1390 : 20~30μL)
16. Centrifuge at 13000rpm for 2 minutes

Primer design used in target mimicry overlapping PCR

A.



B.

1. miR396

   Mature sequence: UUCCACAGCUUUCUUGAACUG

   Forward primer sequence:

   AACAGTTCAAGAACTAAGCTGTGGAAAGCTTCGGTTCCCCTCG

   Reverse primer sequence:

   CTTTCCACAGCTTAGTTCTTGAACTGTTTCTAGAGGGAGATAA

2. miR858

   Mature sequence: UUUCGUUGUCUGUUCGACCUU

   Forward primer sequence:

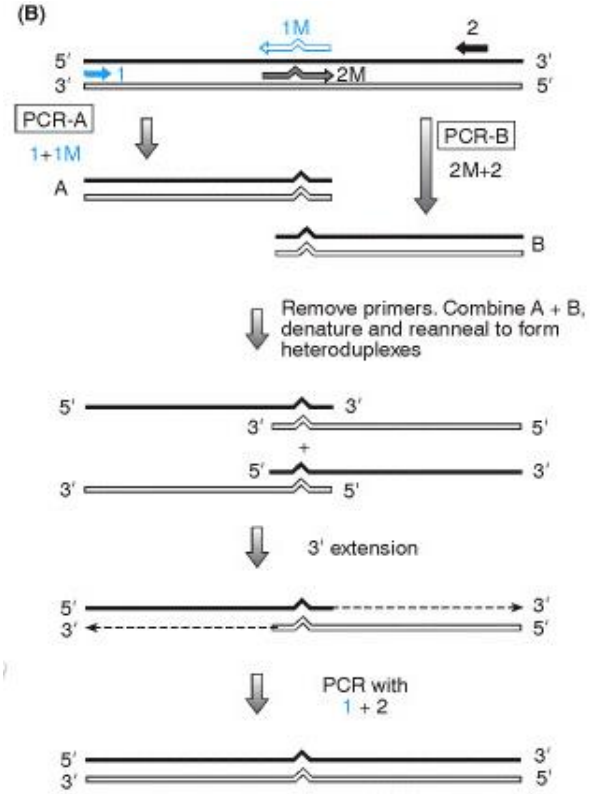
   AAAAGGTCGAACACTAGACAACGAAAAGCTTCGGTTCCCCTCG

   Reverse primer sequence:

   CTTTTCGTTGTCTAGTGTTCGACCTTTTTCTAGAGGGAGATAA

Target mimicry overlapping PCR

A.



(A1 represents Primer1; miRNA RV represents 1M; B2 represents Primer2; miRNA FW represents 2M)

(Origin gene is *IPS1*)

B. PCR-A and PCR-B

1. Arrangement

1.	A1 + miR396 RV
2.	B2 + miR396 FW
3.	A1 + miR858 RV
4.	B2 + miR858 FW

2. PCR materials

Template (*IPS1*/pGEM T-easy)	1μL
5X PCR buffer(BIO-RAD 5X iProof HF Buffer#172-5391)	10μL
10μM FW primer	2.5μL
10μM RV primer	2.5μL
10mM dNTP (GM Cat#GM007)	1μL
iProof High-Fidelity DNA Polymerase (BIO-RAD Cat#172-5301)	0.5μL
ddH_2O	32.5μL
Total	50μL

PCR condition

98℃	30 seconds	
98℃	10 seconds	35 cycles
55℃	30 seconds	
72℃	1 minute	
72℃	5 minutes	
4℃	Pause	

3. Add 6X dye 10 μL to each eppendorf

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

4. Get 30μL to do eletrophoresis in order to confirm the size (1% TAE-Agarose gel, 100 volt, 20 minutes) (Marker: 1 Kb Plus DNA)
5. Mix the other 30μL of two product into one eppendorf

Mix	A1 + miR396 RV
	B2 + miR396 FW
Mix	A1 + miR858 RV
	B2 + miR858 FW

C. Purification of PCR product

(Using Gene-Spin^{TM}-V^{2}, 1-4-3 DNA Extraction Kit Cat# RT-DNA143V$_2$)

1. Add 100μL to each eppendorf in order to dilute the DNA
2. Add 700μL onto the columns
3. Centrifuge for 1 minute at 13000rpm
4. Add the left sample onto the columns
5. Centrifuge for 1 minute at 13000rpm
6. Change a new set of eppendorfs
7. Add 700μL Wash Buffer
8. Centrifuge for 1 minute at 13000rpm
9. Pour off the flow through
10. Centrifuge for 2 minutes at 13000rpm
11. Prepare a new set of eppendorfs and put the columns with purified plasmid on eppendorfs
12. Add 100μL ddH_2O
13. Wait for 2 minutes
14. Centrifuge for 2 minutes to get purified DNA

D. 2^{o}PCR

1. PCR materials

Template (Purified DNA)	1μL
5X PCR buffer (BIO-RAD 5X iProof HF Buffer#172-5391)	10μL
IPS1-A1 (10μM)	2.5μL
IPS1-B2 (10μM)	2.5μL
10mM dNTP (GM Cat#GM007)	1μL
S-taq (BIO-RAD iProof High-Fidelity DNA Polymerase)	0.5μL
ddH_2O	32.5μL
Total	50μL

PCR condition

98℃	30 seconds	
98℃	10 seconds	35 cycles
55℃	30 seconds	
72℃	1 minute	
72℃	5 minutes	
4℃	Pause	

E. PCR product A tailing

F. Get target mimicry insert

1. Add 6X dye 10 μL to each eppendorf

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

2. Get 60μL separated to two wells to do eletrophoresis (1% TAE-Agarose gel, 120 volt, 20 minutes) (Marker: 1 Kb Plus DNA)
3. Purification of DNA from gel

Transform to *E. coli*

1. Add 10ng plasmid to 100μL competent cell
2. Wait for 30 minutes on ice
3. Heat shock (42℃) for 30 seconds and then put it back to the ice immediately for at least 90 seconds
4. Add 1c.c. LB
5. Wait for 1 hour at 37℃ with slight shake
6. Centrifuge for 1 minute at 13000rpm
7. Pour clear supernatant liquid 1c.c., and flush up the bacteria at the bottom
8. Cultivate *E. coli* in the medium at 37℃

Transform to *Agrobacterium tumefaciens* by electroporation

1. Get 1 c.c. *Agro* bacteria liquid (LB)
2. Centrifuge for 5 minutes
3. Pour off supernatant liquid
4. Add 100μL glycerol (10%)
5. Add 100ng plasmid
6. Electroporation (1500V, 200Ω)
7. Add 900μL LB
8. Recovery for 2.5 hours at 28℃
9. Pour clear supernatant liquid 1c.c., and flush up the bacteria at the bottom
10. Cultivate *Agro* in the medium at 28℃

Stock E. coli or Agro

1. Pick up the colony with tips and put it into 5c.c. LB (contain antibiotics)
2. Cultivate *E. coli* for 16 hours at 37℃ while *Agro* for 16 hours at 28℃ with slight shake
3. Get 2c.c. to be stock
   (An eppendorf contains 1c.c. LB and 1c.c. glycerol)
   (Put samples into $N_{2(aq)}$ immediately and storage at -80℃)
4. The left 3c.c. *E. coli* is used to be purified with a view to sequencing, digestion or being template; however, the left 3c.c. *Agro* is used to do dipping

Ligation to T-A vector (pGEM T-easy) and Binary vector (pCambia 1390 with 35s)

1. material

Insert (20~30ng)	1μL	-
Vector (10ng/μL)	1μL	1μL
ddH_2O	3μL	4μL
2X TaKaRa ligation mix (Cat#6023)	5μL	5μL
Total	10μL	10μL

(Ligation without inserts is for control. Before doing colony PCR, we can compare the colonies with and without inserts ligated to know the rate of self-ligated of the plasmids)

2. Wait for overnight at 4℃ or for 1hour at 16℃

_Sal_I and _SacI_ digestion

1. materials

Plasmid	10μL (1~2μg Plasmid DNA)
NEBuffer 4 (Cat# B7004S)	1.5μL
*Sal*I (TaKaRa *Sal*I Cat#1080A)	0.3μL
*Sac*I (TaKaRa *Sac*I Cat#1078A)	0.3μL
ddH_2O	2.9μL
Total	15μL

2.Wait for one hour at 37℃

3. Add 6X dye 3 μL to each eppendorfs

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

4. Eletrophoresis
   (1% TAE-Agarose gel, 120 volt, 20 minutes) (Marker: 1 Kb Plus DNA)
5. Purification of DNA from gel

Murashige and Skoog Basal Salt medium for growing of seedlings

MS salt (Duchefa)	1/2X
Phyto Agar (Duchefa Phyto agar, Cat#P1003)	0.8%

Bacteria medium

yeast extract	0.5%
Tryptone	1%
NaCl	1%
pH	7.0

Antibiotics used in medium for selection

Antibiotics	Species	Concentration
Hygromycin & Kanamycin	pCambia 1390	50μg/ml
Hygromycin	T-DNA	50μg/ml
Ampicillin	pGEM T-easy	100μg/ml
Gentamicin	*Agro*	50μg/ml

Dipping

1. Materials

Bacteria liquid medium (LB)	5ml
1/2 MS salt medium (Must be added before Silwet-L-77)	10ml
Silwet-L-77	5μL
Total	20.005ml

2. Dip the flower buds with dropper until they are soaking wet. Loosely cover the plants with saran wrap for 2-3 days.
3. Do not water the plants after dip the plants

Southern blotting for checking the number of the inserts

A. Digestion and Eletrophoresis

1. materials

Genomic DNA	13.2μL (1-7μg genomic DNA)
NEBuffer 4 (Cat# B7004S)	1.5μL
EcoRV-HF® , NEB, Cat# R3195	0.3μL
Total	15μL

2.Wait for one hour at 37℃

3. Add 6X dye 3 μL to each eppendorfs

6X dye	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	(w/v) sucrose in H_2O	40%

4. Eletrophoresis

(1% TAE-Agarose gel, 70 volt, 90 minutes) (Marker: 1 Kb Plus DNA)

B. Synthesis of DIG- labeled probe by PCR

1. PCR materials

	DIG-labeled (μL)	Non-labeled (μL)
Genomic DNA (5ng/μL)	2	2
Super Therm Taq DNA polymerase (Medox-Bio® Cat# MX-0647)	0,1	0.1
FW Primer (10μM)	2.5	2.5
RV Primer (10μM)	2.5	2.5
Digoxigenin-11-dUTP, Roche, Cat#11093088910	1	-
dNTP (10mM) (GM Cat#GM007)	1	1
R. Buffer (10X) with 15mM $MgCl_2$ (Cat#JMR-420)	5	5
ddH_2O	35.9	36.9
Total	50	50

PCR condition

95℃	5 minutes	
95℃	30 seconds	35 cycles
55℃	30 seconds	
72℃	1.5 minutes	
72℃	7 minutes	
4℃	Pause	

2. Get 2μL to conduct (1% TAE-Agarose gel, 120 volt, 15 minutes) (Checking the quantity of probe)
3. Purification of PCR product

C. Denature DNA

1. Add 100-200mL HCl (0.25M) to a plastic box with a piece of gel
2. Spin it at 60rpm for 5 minutes at RT
3. Pour off the buffer
4. Repeat step 1-3 again
5. Wash the gel with ddH_2O
6. Add 100-200mL NaOH solution (0.5M) to the plastic box
7. Spin it at 60rpm for 10-15 minutes at RT
8. Pour off the buffer
9. Repeat step 6-8 again

10. Wash the gel with ddH_2O
11. Add 100-200mL Tris(0.5M)-NaCl(1.5M) buffer (pH 7.4) to the plastic box
12. Spin it at 60rpm for 10-15 minutes at RT
13. Pour off the buffer
14. Repeat step 11-13 again

D. Transfer by vacuum blotting (VacuGene XL Vacuum blotting System)

1. Keep the gels and membranes wet with 10X SSC
2. Keep the pressure up to 100 mbar
3. Transfer for 1hr
4. UV-crosslink for about 2 minutes (About 2400 J)

E. Hybridization

1. Pre-hybridize for 2hr at 12rpm at 60℃
   (FastHyb-Hybridization Solution, BioChain, Cat#L1031250)
2. Heat the probe solution at 95℃ for 5 minutes and place it onto the ice quickly
3. Wait for 5 minutes
4. Add the probe solution in the plastic tube to the hybridization buffer and spin it at 12 rpm at 60℃ overnight

F. Wash

1. Add Wash I buffer 100mL to a plastic box
2. Move the membrane into the buffer and spin it at 60rpm for 5 minutes at RT
3. Pour off the buffer
4. Add Wash I buffer 100mL to the plastic box and spin it at 60rpm for 5 minutes at RT again
5. Pour off the buffer
6. Add Wash II buffer 100mL to the plastic box (Heated to 60℃)
7. Spin it at 60rpm for 5minutes at 60℃
8. Pour off the buffer

9. Repeat step 6-8 again

Wash buffer I		Wash buffer II	
20X SSC	10mL	20X SSC	5mL
10%SDS	10mL	10%SDS	10mL
Add H_2O to 1L		Add H_2O to 1L	

(SSC 20X buffer, Bioman, Cat# SSC205000)
(SDS 10% solution, Bioman, Cat# SDS101000)

G. Preparation for detection

1. Dilute 10X Maleic acid buffer into 1X Maleic acid buffer

10X Maleic acid buffer (pH is adjusted to 7.5 with NaOH)	
Maleic acid	116.08g
NaCl	58.44g
Add H_20 to 1 L	

2. Mix 45mL 1X Maleic acid buffer and 5mL 10X Blocking buffer (5g Blocking Reagent, Roche, Cat#11096176001) to make 1X Blocking buffer
3. Add Tween 20 wash buffer 100mL to the plastic box with the membrane in

Tween 20 wash buffer	
Tween 20 (100%)	0.3%
In 1X Maleic acid buffer	

(TWEEN® 20, SIGMA, Cat#P9416 SIGMA)

4. Spin it at 60rpm for 5minutes at RT
5. Pour off the buffer
6. Add 15mL 1X Blocking buffer
7. Spin it at 60rpm for at least 30 minutes but no more than 3hrs at RT
8. Pour off the buffer
9. Add 20mL 1X Blocking buffer containing 2μL anti-DIG-AP (Anti-Digoxigenin-AP, Fab fragments, Cat#11093274910)
10. Spin it at 60rpm for at least 30 minutes but no more than 3hrs at RT
11. Pour off the buffer
12. Add 100-200mL 1X Maleic acid buffer to the plastic box
13. Spin it at 60rpm for 15 minutes at RT
14. Pour off the buffer
15. Repeat step 12-14 again
16. Add 10mL detection buffer to the plastic box

Detection buffer (1L)	
1M Tris-HCl (pH 9.5)	100mL
5M NaCl	20mL
H_2O	880mL

17. Spin it at 60rpm for 5 minutes at RT
18. Pour off the buffer
19. Dilute 20μL CDP (CDP-Star® , SIGMA, Cat#C0712) into 2mL detection buffer
20. Add the buffer onto the membrane and put it into a plastic bag

H. Detection

1. Expose the film to the membrane for 30 minutes to several hours
   (Amersham Hyperfilm MP, GE Healthcare Life Sciences, Cat# 28-9068-42)

Quantification of anthocyanin

1. Measure the weight of each eppendorf
2. Get fresh 30-100 seedlings dried by tissue papers into each eppendorf
3. Measure the eppendorf filledwith tissue again, and we can know the weight of seedlings
4. Put the samples into N_2(l)
4. Gring the tissue in N_2(l) and do not pick up the pestle
5. Immerse the powder in 1% HCl, 18% 1-propanol and use the solution to clean the pestle
6. Put the sample in boil water for 60 seconds
7. Shake and centrifugate the sample for 5 minutes at 13000 rpm
8. Put the sample in boil water for 90 seconds
6. Centrifuge for 15 minutes at 13000 rpm and get supernatant
7. Measure the sample by Bio-Tek PowerWave X340
8. A535-correct = A535-A650

Quantification of chlorophyll

1. Measure the weight of each eppendorf
2. Get fresh 30-100 seedlings dried by tissue papers into each eppendorf
3. Measure the eppendorf filledwith tissue again, and we can know the weight of seedlings
4. Immerse the seedlings in 100% dimethyl formamide at the ratio of 2-7% for 24 - 48 shours
5. Centrifuge for 15 minutes at 13000 rpm and get supernatant
6. Measure the sample by Bio-Tek PowerWave X340
7. Ctotal = Ca+Cb=7.04xA664+20.27xA647

Plasmid sequencing

1.

Category	Amount
Plasmid	1μg/8μL
PCR product	100ng/8μL

2. Primers of pGEM T-easy sequencing

Primer name	Sequence	Purpose
M13F	GTAAAACGACGGCCAGT	For sequencing

3. Primers of *35S::*pCambia 1390 sequencing

Primer name	Sequence	Purpose
35S-5'-seq	CCACTATCCTTCGCAAGACCC	For sequencing

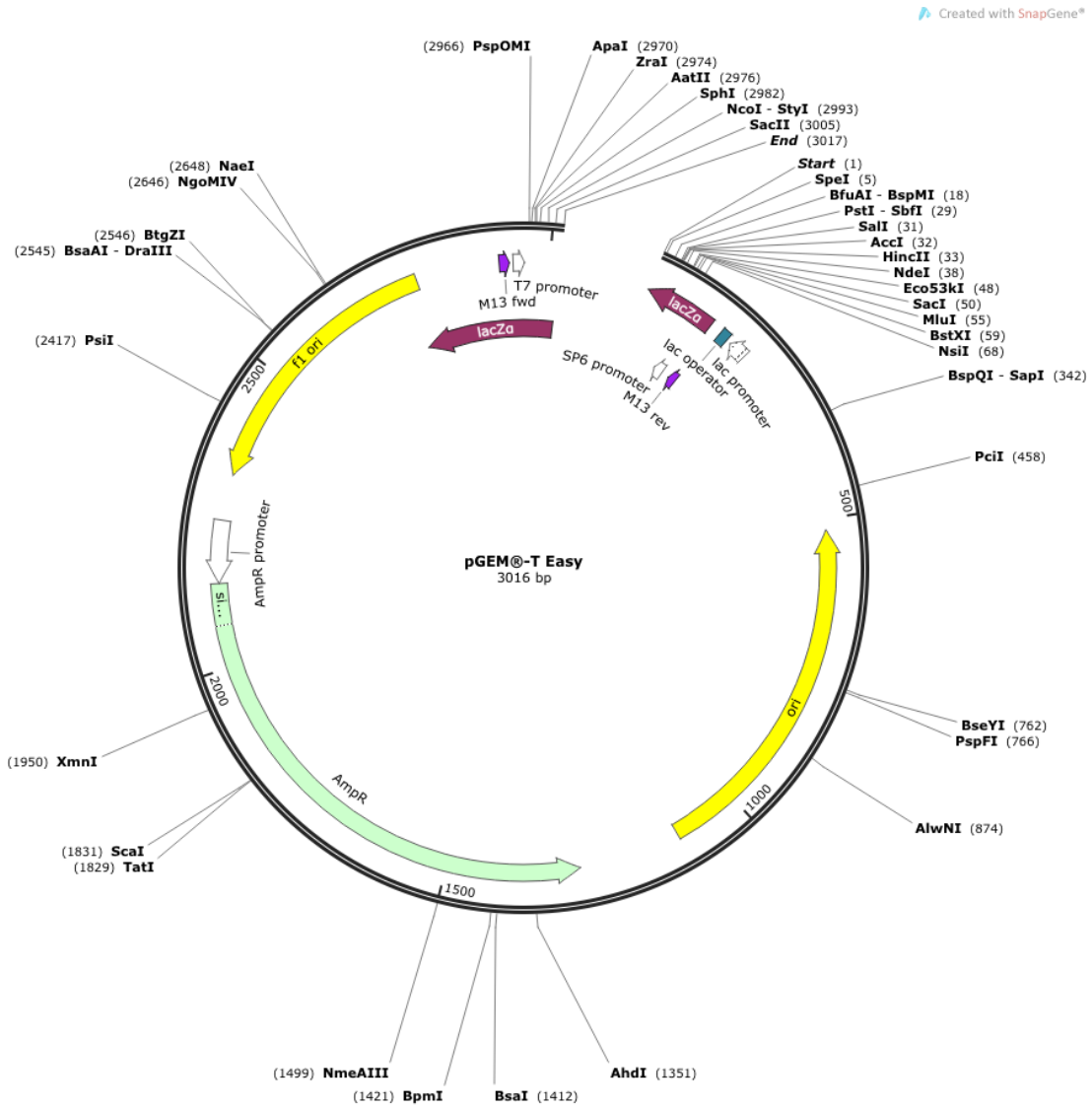
DNA Size

Category	miR396	miR858
pGEM T-easy	3016 bp	
*35S::LUC2/*pCambia 1390 /after digestion	11428 bp /9775 bp or 9729 bp	
target mimicry insert/with enzyme site	522 bp /528bp	522 bp /528bp
miRNA overexpression insert/with enzyme site	935 bp /941bp	1009 bp /1015bp

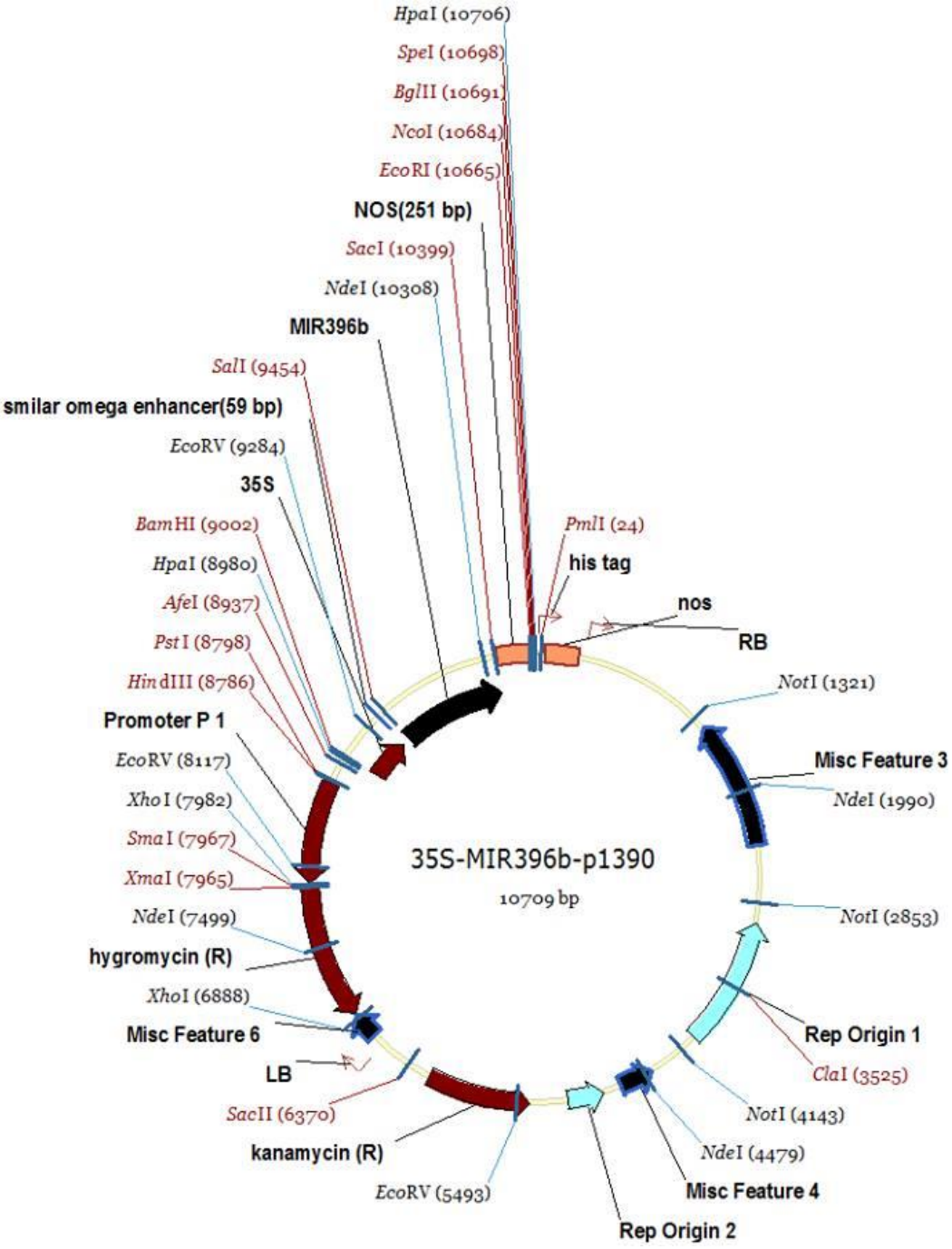
Construct list

Category	miR396	miR858
Preparation	*IPS1*/pGEM T-easy	
target mimicry : pGEM T-easy	*MIM396*/pGEM T-easy	*MIM858*/pGEM T-easy
target mimicry : *35S::*pCambia 1390	*35S::MIM396*/ pCambia 1390	*35S::MIM858*/ pCambia 1390
miRNA overexpression : pGEM T-easy	*MIR396*/pGEM T-easy	*MIR858*/pGEM T-easy
miRNA overexpression : *35S::*pCambia 1390	*35S::MIR396*/ pCambia 1390	*35S::MIR858*/ pCambia 1390

pGEM T-easy (As a T-A vector)



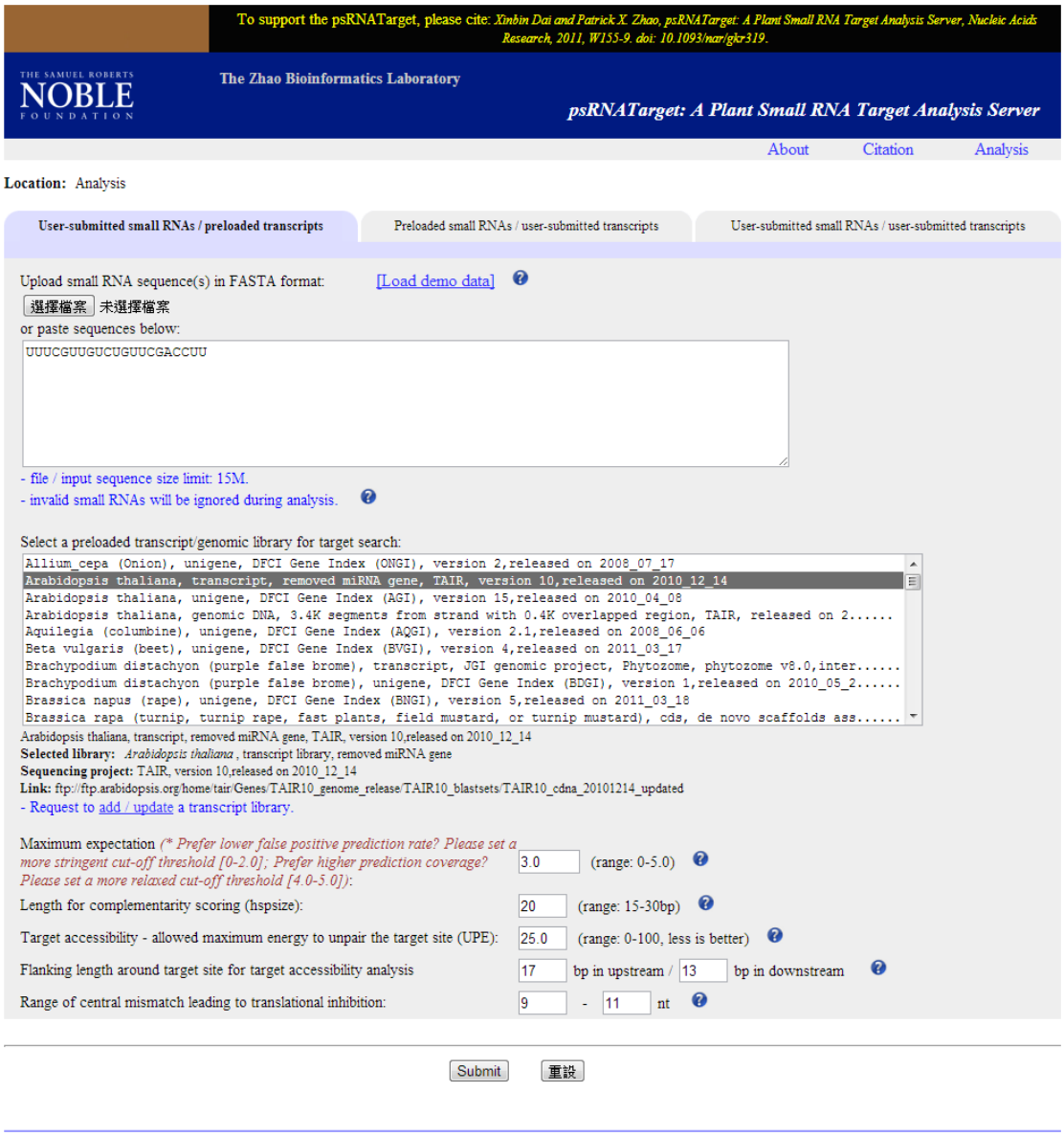
***35S::MIR396B*/pCambia 1390 (For it for example)**



Predict targets of miRNAs

(By using “psRNATarget: A Plant Small RNA Target Analysis Server”)

1. Operator interface



2. List of predicted targets of miRNAs

miRNA Acc.	Target Acc.	Expectation (E)	Target Accessibility (UPE)	Inhibition
MIR396A	*AT3G52910.1*	2	14.357	Cleavage
MIR396A	*AT5G43060.1*	2	17.343	Cleavage
MIR396A	*AT5G01370.1*	2	19.954	Cleavage
MIR396A	*AT2G34530.1*	2	21.186	Cleavage
MIR396A	*AT2G34530.2*	2	21.186	Cleavage
MIR396A	*AT2G36400.1*	2	22.724	Cleavage
MIR396A	*AT3G14110.1*	2.5	16.108	Translation
MIR396A	*AT3G14110.2*	2.5	16.108	Translation
MIR396A	*AT3G14110.3*	2.5	16.108	Translation
MIR396A	*AT1G35370.1*	2.5	17.913	Translation
MIR396A	*AT5G57590.1*	2.5	18.079	Cleavage
MIR396A	*AT1G80060.1*	2.5	19.538	Cleavage
MIR396A	*AT4G12050.1*	3	16.311	Cleavage
MIR396A	*AT2G15630.1*	3	16.675	Cleavage
MIR396A	*AT5G58980.1*	3	20.772	Cleavage
MIR858A	*AT3G08500.1*	1.5	14.533	Cleavage
MIR858A	*AT5G35550.1*	1.5	20.534	Cleavage
MIR858A	*AT1G66230.1*	2	7.972	Cleavage
MIR858A	*AT4G12350.1*	2	10.793	Cleavage
MIR858A	*AT5G49330.1*	2	11.727	Cleavage
MIR858A	*AT1G06180.1*	2	13.241	Cleavage
MIR858A	*AT1G79180.1*	2	14.251	Cleavage
MIR858A	*AT2G47460.1*	2	15.142	Cleavage
MIR858A	*AT2G26950.1*	2	17.7	Cleavage
MIR858A	*AT4G09460*	2.5	11.378	Cleavage
MIR858A	*AT1G34670.1*	2.5	14.703	Cleavage
MIR858A	*AT3G11440.1*	2.5	14.874	Cleavage
MIR858A	*AT5G14340.1*	2.5	16.906	Cleavage
MIR858A	*AT1G71030.1*	2.5	20.458	Translation
MIR858A	*AT1G17760.1*	2.5	21.667	Cleavage
MIR858A	*AT3G46130.1*	3	12.123	Cleavage
MIR858A	*AT3G46130.2*	3	12.123	Cleavage
MIR858A	*AT3G46130.3*	3	12.123	Cleavage
MIR858A	*AT3G46130.4*	3	12.123	Cleavage

MIR858A	*AT5G05800.1*	3	21.591	Cleavage
MIR858A	*AT5G05800.2*	3	21.591	Cleavage

(Take miR396a/b, miR858a/b for examples)

(Targets with yellow blanks are selected to be conducted Real-Time PCR on)

(*GRF*1, 2, 3,7 ,8 ,9 are selected to calculate the Pearson correlation coefficient with the *MIR396*s due to the support of the literature review.)

Primers list used in Real-Time PCR

Primer name	Sequence	Purpose
AT1G80060 FW	CCAGGAGCACAAGACGAGTT	Real-Time PCR
AT4G09460 FW	TCAAAACGATGCCGTTGAGT	Real-Time PCR
AT5G05800 FW	GATCGGCTTCTTCCAGCCAC	Real-Time PCR
AT4G37740 FW	TGTTCATGTTCTTGGGTCGGT	Real-Time PCR
AT2G36400 FW	CCATACGAGTCCCACATCGG	Real-Time PCR
AT5G53660FW	CCGATGTCTACCACACCTGG	Real-Time PCR
AT1G80060 RV	CGACACTCGGGTCCTTTTCT	Real-Time PCR
AT4G09460 RV	TAACACTCCCCATTCTGCCG	Real-Time PCR
AT5G05800 RV	TCTGTTCTCCAAAACTCCGGG	Real-Time PCR
AT4G37740 RV	CGTTGCAAGCAATCCTCACC	Real-Time PCR
AT2G36400 RV	CTGAGCTCATGGGGCTTGAA	Real-Time PCR
AT5G53660 RV	TCACTCTAGACGGGGACGAG	Real-Time PCR

Primer name	Sequence	Purpose
UBQ10-ABI-1	5'AGAAGTTCAATGTTTCGTTTCATGTAA3'	Real-Time PCR
UBQ10-ABI-2	5'GAACGGAAACATAGTAGAACACTTATTCA3'	Real-Time PCR

Primers list used in target mimicry

Primer name	Sequence	Purpose
MIM858-RV	CTTTTCGTTGTCTAGTGTTCGACCTTTTTCTAGAGGGAGATAA	For target mimicry of miR858
MIM858-FW	AAAAGGTCGAACACTAGACAACGAAAAGCTTCGGTTCCCCTCG	For target mimicry of miR858
MIM396-RV	CTTTCCACAGCTTAGTTCTTGAACTGTTTCTAGAGGGAGATAA	For target mimicry of miR396
MIM396-FW	AACAGTTCAAGAACTAAGCTGTGGAAAGCTTCGGTTCCCCTCG	For target mimicry of miR396
*Sal*I-*IPS1*-A1	GTGTCGACAAGAAAAATGGCCATCCCCTAGC	For target mimicry
S*ac*I-*IPS1*-B2	CTGGAGCTCGAGGAATTCACTATAAAGAGAATCG	For target mimicry

(Red part is *Sal*I enzyme-cut site; Yellow is part is *Sac*I enzyme-cut site enzyme-cut site)

Primers list used in Cloning

Primer name	Sequence	Purpose
*Sal*I-*MIR396b*-FW	GTAGTCGACACTATCTAAGC ACCACTAAG	For cloning *MIR396b* gene
*Sal*I-*MIR858a*-FW	GTAGTCGACGGTACGGTACA GTGTGTGTTG	For cloning *MIR858a* gene
*Sac*I-*MIR396a*-RV	CTGGAGCTCTCACAGAGTACC AATATAAT	For cloning *MIR858a* gene
*Sac*I-*MIR858a*-RV	CTGGAGCTCGGAACCCTCTCA CGTCAATATGTATATTAAC	For cloning *MIR858a* gene
*Sal*I-*IPS1*-A1	GTGTCGACAAGAAAAATGGCC ATCCCCTAGC	For cloning *IPS1*/ target mimicry inserts
*Sac*I-*IPS1*-B2	CTGGAGCTCGAGGAATTCACT ATAAAGAGAATCG	For cloning *IPS1*/ target mimicry inserts

(Red part is *Sal*I enzyme-cut site enzyme-cut site; Yellow is part is *Sac*I enzyme-cut site enzyme-cut site)

Primers list used in colony PCR

Primer name	Sequence	Purpose
35S-5'-seq	CCACTATCCTTCGCAAGACCC	As FW primer in colony PCR for *35S::*pCambia 1390
Nos terminator	GGCAACAGGATTCAATCTTA(20)	As RV primer in colony PCR of overexpression line for *35S::*pCambia 1390
*Sal*I-*IPS1*-A1	GTGTCGACAAGAAAAATGGCC ATCCCCTAGC	As FW primer in colony PCR of target mimicry line for pGEM T-easy
*Sac*I-*IPS1*-B2	CTGGAGCTCGAGGAATTCAC TATAAAGAGAATCG	As RV primer in colony PCR of target mimicry line for *35S::*pCambia 1390 / As RV primer in colony PCR of target mimicry line for pGEM T-easy
*Sal*I-*MIR396b*-FW	GTAGTCGACACTATCTAAGC ACCACTAAG	As FW primer in colony PCR of overexpression line for pGEM T-easy
*Sal*I-*MIR858a*-FW	GTAGTCGACGGTACGGTACA GTGTGTGTTG	As FW primer in colony PCR of overexpression line for pGEM T-easy
*Sac*I-*MIR396a*-RV	CTGGAGCTCTCACAGAGTAC CAATATAAT	As RV primer in colony PCR of overexpression line for pGEM T-easy
*Sac*I-*MIR858a*-RV	CTGGAGCTCGGAACCCTCTC ACGTCAATATGTATATTAAC	As RV primer in colony PCR of overexpression line for pGEM T-easy

(Red part is *Sal*I enzyme-cut site enzyme-cut site; Yellow is part is *Sac*I enzyme-cut site)

Primers list used in 5'RACE

Primer name	Sequence	Expected amplicon
At4g09460-RACE-1	ATCCTCGTCCGTCGTCGTGC	379
At4g09460-RACE-2	AAAAGAATACTCAACGGCATCG	292
At5g05800-RACE-1	GATTCTCAAACTGGGCTTCTTCA	391
At5g05800-RACE-2	GCTCACTACCTTGTTCGACCTC	302

Genotyping list

miRNA	Target	T-DNA line	LP	RP	Amplicon (WT)	Amplicon (LBb1.3+RP)
miR858a	*AT4G09460*	SALK_074789C	TCTCTTCCAATCAGCTGCTTC	TTGTTTTGCAGATGGTCATTG	1188	709
miR396a mutant		SALK_064046	TGATTATGGAATCAATCACGC	AATTTCCTCCACCCAATTTTG	1066	713
miR396a mutant		SALK_064047	TGATTATGGAATCAATCACGC	AATTTCCTCCACCCAATTTTG	1066	713

Primer used in Southern blotting

Category	Name	Sequence	Purpose
FW Primer	NPT II-S1	AAGAACTCGTCAAGAAGGCG	Southern blot
RV Primer	NPT II-AS1	AGATGGATTGCACGCAGGTT	Southern blot

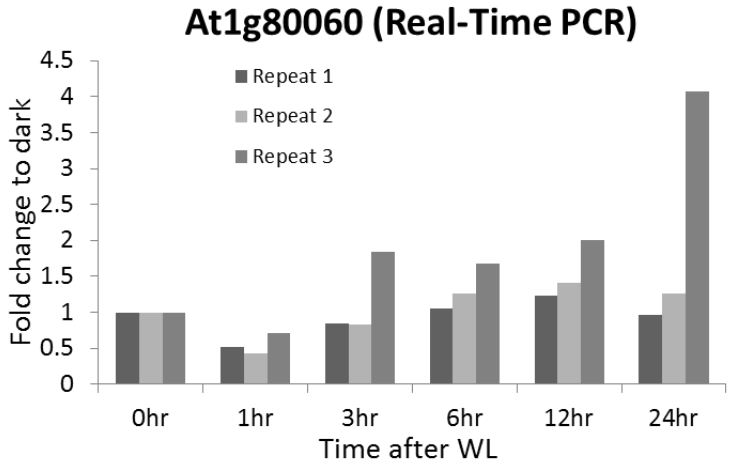
Original sRNA NGS data (read counts)

miRMA	0hr	1 hr	3 hr	6 hr	12 hr	24 hr
miR159a	1770.58	3952.5	3698	6380.75	2178.75	3409.33
miR159b	355.08	653.5	710	1261.75	517.75	613.83
miR159c	147.08	143.5	244	240.25	203.25	144.83
miR160a	1573.5	801.99	1295.49	1171.5	1124.01	776.01
miR160b	1135.5	576.99	1014.99	874.5	653.01	228.51
miR160c	1572	789.99	1274.49	1170	1121.01	765.51
miR162a	2837	6613	2224	5777	2811	1565
miR162b	2839	6613	2222	5777	2811	1565
miR394a	408	327	231	251	55	15
miR394b	410	327	229	251	55	17
miR396a	678.5	647.5	1009.5	586.5	622	848
miR396b	505.5	1372.5	621.5	1302.5	692	721
miR858a	51	259	134	287	71	101
miR858b	9.5	22.5	19	31.5	9.5	12
miR869	139	112	141	182	210	480

Original mRNA NGS data (RPKM)

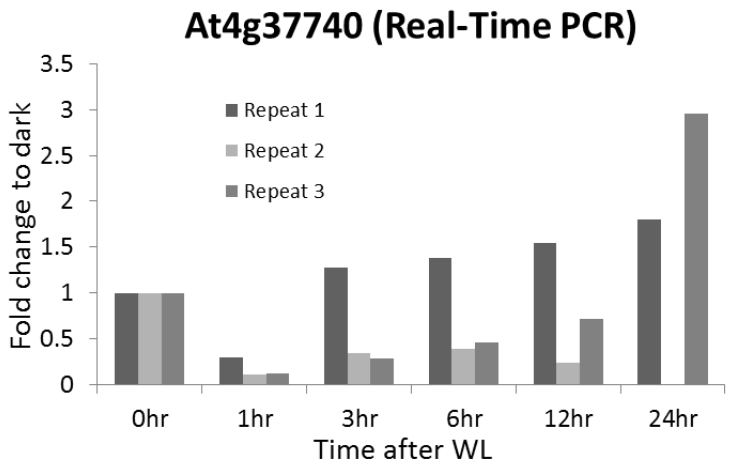
miRNA	Target	Gene	0hr	1hr	3hr	6hr	12hr	24hr
miR396	*AT1G80060.1*	Ubiquitin-like	4.70318	2.17561	2.56869	1.77353	2.90148	4.39254
miR396	*At4g37740.1*	*GRF2*	22.4398	5.80687	6.39078	7.73999	8.25023	4.64976
miR396	*AT2G36400.1*	*GRF3*	19.6936	6.70783	16.0285	14.5603	12.2283	8.30605
miR396	*At5g53660.1*	*GRF7*	53.8783	1.52058	0.291602	0.515996	0.15539	0
miR858	*AT4G09460*	*MYB6*	26.9178	10.2494	8.52513	10.85	12.5418	13.5734
miR858	*AT5G05800.1*	unknown	2.18518	5.74455	4.18334	4.84014	1.46886	3.79274
miR858	*AT5G05800.2*	unknown	10.4541	4.58218	6.00288	4.54879	10.1916	12.2304

Real-Time PCR data



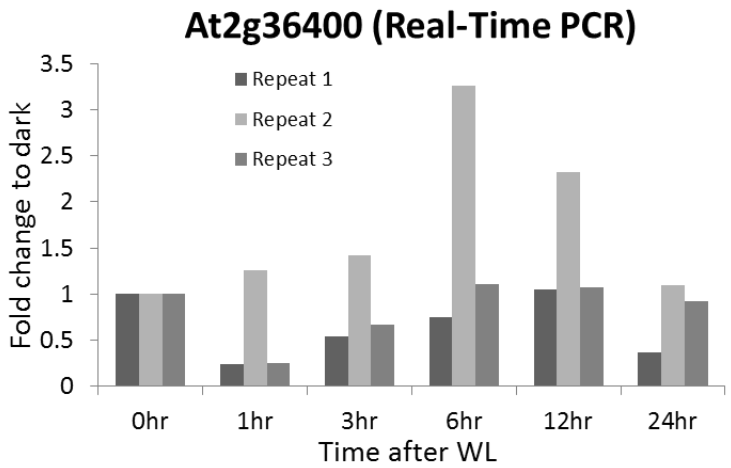
Sample Name	Target Name	2^-dCt	Normalized	AVG	STD
Biological Repeat 1					
WL 0h	At1g80060	0.0014829	1.134324	1	0.1201751
WL 0h	At1g80060	0.0011801	0.9026742		
WL 0h	At1g80060	0.001259	0.9630019		
WL 1h	At1g80060	0.0006914	0.5288648	0.5244586	0.0268811
WL 1h	At1g80060	0.0007175	0.5488644		
WL 1h	At1g80060	0.000648	0.4956467		
WL 3h	At1g80060	0.000854	0.6532435	0.8405598	0.1807131
WL 3h	At1g80060	0.0013254	1.0138528		
WL 3h	At1g80060	0.0011172	0.8545832		
WL 6h	At1g80060	0.0013784	1.0543496	1.0465206	0.0342759
WL 6h	At1g80060	0.001407	1.0762047		
WL 6h	At1g80060	0.0013191	1.0090075		
WL 12h	At1g80060	0.0015767	1.2060566	1.2348147	0.0560901
WL 12h	At1g80060	0.0016988	1.2994514		
WL 12h	At1g80060	0.0015674	1.198936		
WL 24h	At1g80060	0.0014128	1.0806683	0.9582508	0.1241316
WL 24h	At1g80060	0.0012571	0.9616108		
WL 24h	At1g80060	0.0010883	0.8324734		
Biological Repeat 2					
WL 0h	At1g80060	0.0020571	1.021501	1	0.0187021
WL 0h	At1g80060	0.0019887	0.9875041		
WL 0h	At1g80060	0.0019957	0.9909948		
WL 1h	At1g80060	0.0009297	0.4616345	0.4282425	0.028928
WL 1h	At1g80060	0.0008273	0.4107992		

WL 1h	At1g80060	0.0008303	0.4122938		
WL 3h	At1g80060	0.0017132	0.8506933	0.82344	0.1336787
WL 3h	At1g80060	0.0013659	0.6782346		
WL 3h	At1g80060	0.0018958	0.9413919		
WL 6h	At1g80060	0.0024607	1.221907	1.2604969	0.0490698
WL 6h	At1g80060	0.0026497	1.3157218		
WL 6h	At1g80060	0.0025049	1.243862		
WL 12h	At1g80060	0.0028665	1.4234062	1.4102724	0.1192003
WL 12h	At1g80060	0.0030658	1.5223618		
WL 12h	At1g80060	0.0025879	1.2850492		
WL 24h	At1g80060	0.0030633	1.5211333	1.2612098	0.323569
WL 24h	At1g80060	0.0018101	0.8988115		
WL 24h	At1g80060	0.0027462	1.3636845		
Biological Repeat 3					
WL 0h	At1g80060	0.0004368	1.1081262	1	0.0977996
WL 0h	At1g80060	0.0003617	0.917718		
WL 0h	At1g80060	0.000384	0.974156		
WL 1h	At1g80060	0.000301	0.76411	0.705063	0.087226
WL 1h	At1g80060	0.000294	0.746204		
WL 1h	At1g80060	0.000238	0.604875		
WL 3h	At1g80060	0.000714	1.811083	1.8482111	0.106131
WL 3h	At1g80060	0.000776	1.967918		
WL 3h	At1g80060	0.000696	1.765633		
WL 6h	At1g80060	0.000778	1.974655	1.6783414	0.306869
WL 6h	At1g80060	0.000669	1.698463		
WL 6h	At1g80060	0.000537	1.361907		
WL 12h	At1g80060	0.000671	1.703113	2.0067878	0.265797
WL 12h	At1g80060	0.000836	2.120095		
WL 12h	At1g80060	0.000866	2.197155		
WL 24h	At1g80060	0.001602	4.06503	4.0709054	0.033628
WL 24h	At1g80060	0.001593	4.040602		
WL 24h	At1g80060	0.001619	4.107084		



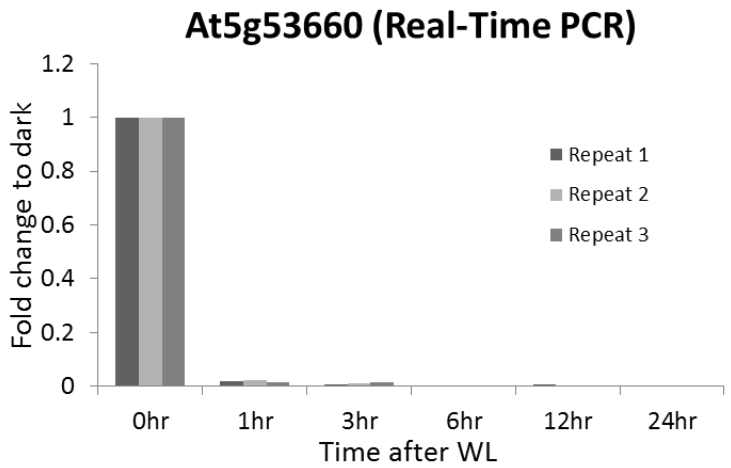
Sample Name	Target Name	2^-dCt	Normalized	AVG	STD
Biological Repeat 1					
WL 0h	GRF2	0.00404	1.008576	1	0.070646
WL 0h	GRF2	0.003707	0.925457		
WL 0h	GRF2	0.004269	1.065967		
WL 1h	GRF2	0.001416	0.353639	0.2982998	0.049859
WL 1h	GRF2	0.001139	0.28438		
WL 1h	GRF2	0.001029	0.25688		
WL 3h	GRF2	0.005567	1.389989	1.280345	0.351821
WL 3h	GRF2	0.006265	1.564288		
WL 3h	GRF2	0.003552	0.886758		
WL 6h	GRF2	0.004987	1.245048	1.3817212	0.330043
WL 6h	GRF2	0.004574	1.141969		
WL 6h	GRF2	0.007042	1.758147		
WL 12h	GRF2	0.006217	1.552218	1.5482159	0.355407
WL 12h	GRF2	0.007616	1.901605		
WL 12h	GRF2	0.004769	1.190825		
WL 24h	GRF2	0.008931	2.229735	1.8033863	0.375156
WL 24h	GRF2	0.006635	1.656638		
WL 24h	GRF2	0.006103	1.523786		
Biological Repeat 2					
WL 0h	GRF2	0.013607	0.941328	1	0.067499
WL 0h	GRF2	0.014237	0.984903		
WL 0h	GRF2	0.015522	1.073769		
WL 1h	GRF2	0.00135	0.093402	0.104812	0.023727
WL 1h	GRF2	0.001909	0.132089		
WL 1h	GRF2	0.001286	0.088945		

WL 3h	GRF2	0.005979	0.413599	0.3482046	0.085754
WL 3h	GRF2	0.005492	0.379901		
WL 3h	GRF2	0.00363	0.251114		
WL 6h	GRF2	0.005728	0.396232	0.3911687	0.012321
WL 6h	GRF2	0.005784	0.400151		
WL 6h	GRF2	0.005451	0.377123		
WL 12h	GRF2	0.003398	0.235062	0.2426227	0.020313
WL 12h	GRF2	0.00384	0.265631		
WL 12h	GRF2	0.003284	0.227174		
WL 24h	GRF2	0.006042	0.417976	0.3756902	0.042203
WL 24h	GRF2	0.005428	0.375523		
WL 24h	GRF2	0.004822	0.333571		
Biological Repeat 3					
WL 0h	GRF2	0.001472	0.889134	1	0.263662
WL 0h	GRF2	0.002154	1.300992		
WL 0h	GRF2	0.001341	0.809874		
WL 1h	GRF2	0.000227	0.136994	0.1195894	0.033091
WL 1h	GRF2	0.000232	0.140346		
WL 1h	GRF2	0.000135	0.081429		
WL 3h	GRF2	1.5E-05	0.00903	0.2825016	0.116152
WL 3h	GRF2	0.00083	0.501369		
WL 3h	GRF2	0.000558	0.337106		
WL 6h	GRF2	0.000121	0.073072	0.4568191	0.065285
WL 6h	GRF2	0.001151	0.694856		
WL 6h	GRF2	0.000998	0.602529		
WL 12h	GRF2	0.001877	1.133597	0.7172314	0.047731
WL 12h	GRF2	0.000787	0.475297		
WL 12h	GRF2	0.000899	0.5428		
WL 24h	GRF2	0.004559	2.753136	2.954091	0.178757
WL 24h	GRF2	0.005126	3.095396		
WL 24h	GRF2	0.00499	3.013741		



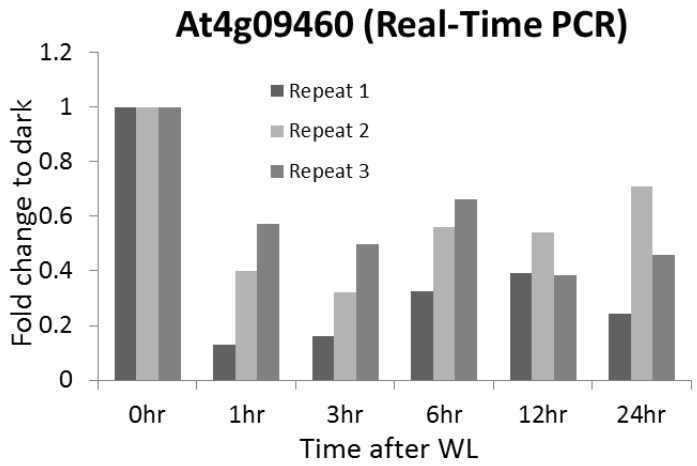
Sample Name	Target Name	2^-dCt	Normalized	AVG	STD
Biological Repeat 1					
WL 0h	GRF3	0.0092	1.137631	1	0.124975
WL 0h	GRF3	0.007834	0.968762		
WL 0h	GRF3	0.007226	0.893607		
WL 1h	GRF3	0.001879	0.232329	0.2401308	0.007958
WL 1h	GRF3	0.002007	0.248237		
WL 1h	GRF3	0.001939	0.239827		
WL 3h	GRF3	0.00398	0.492158	0.545468	0.049403
WL 3h	GRF3	0.004769	0.589707		
WL 3h	GRF3	0.004484	0.554539		
WL 6h	GRF3	0.006514	0.80546	0.7500948	0.048014
WL 6h	GRF3	0.005862	0.724937		
WL 6h	GRF3	0.005822	0.719888		
WL 12h	GRF3	0.008222	1.016756	1.0546413	0.040527
WL 12h	GRF3	0.008874	1.097374		
WL 12h	GRF3	0.008489	1.049794		
Biological Repeat 2					
WL 24h	GRF3	0.003335	0.41234	0.3649078	0.047355
WL 24h	GRF3	0.00295	0.364753		
WL 24h	GRF3	0.002569	0.31763		
WL 0h	GRF3	0.004453	0.945057	1	0.12549
WL 0h	GRF3	0.004294	0.911352		
WL 0h	GRF3	0.005388	1.14359		
WL 1h	GRF3	0.002667	0.566082	0.560708	0.02113
WL 1h	GRF3	0.002726	0.578632		
WL 1h	GRF3	0.002532	0.53741		

WL 3h	GRF3	0.006386	1.355337	1.22437	0.18767
WL 3h	GRF3	0.006165	1.308406		
WL 3h	GRF3	0.004756	1.009368		
WL 6h	GRF3	0.007461	1.583513	1.668919	0.239753
WL 6h	GRF3	0.00699	1.483563		
WL 6h	GRF3	0.009139	1.93968		
WL 12h	GRF3	0.010041	2.131038	2.134082	0.074817
WL 12h	GRF3	0.010414	2.210375		
WL 12h	GRF3	0.00971	2.060834		
WL 24h	GRF3	0.007915	1.67986	1.413155	0.412976
WL 24h	GRF3	0.007643	1.622149		
WL 24h	GRF3	0.004417	0.937457		
Biological Repeat 3					
WL 0h	GRF3	0.009176	1.102046	1	0.154865
WL 0h	GRF3	0.00896	1.076151		
WL 0h	GRF3	0.006842	0.821803		
WL 1h	GRF3	0.001718	0.20634	0.2485012	0.047074
WL 1h	GRF3	0.001997	0.239871		
WL 1h	GRF3	0.002492	0.299293		
WL 3h	GRF3	0.006085	0.730817	0.666759	0.105129
WL 3h	GRF3	0.006028	0.72403		
WL 3h	GRF3	0.004541	0.54543		
WL 6h	GRF3	0.00915	1.098949	1.1073994	0.060524
WL 6h	GRF3	0.008755	1.051545		
WL 6h	GRF3	0.009756	1.171705		
WL 12h	GRF3	0.007105	0.853388	1.0691013	0.186913
WL 12h	GRF3	0.009749	1.170873		
WL 12h	GRF3	0.00985	1.183043		
WL 24h	GRF3	0.008018	0.96296	0.9254042	0.062927
WL 24h	GRF3	0.007997	0.960497		
WL 24h	GRF3	0.0071	0.852756		



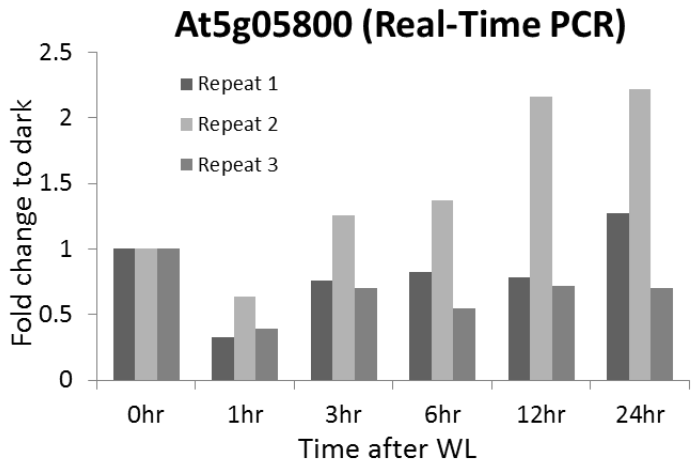
Sample Name	Target Name	2^-dCt	Normalized	AVG	STD
Biological Repeat 1					
WL 0h	GRF7	0.06619	0.699657	1	0.195705
WL 0h	GRF7	0.081512	0.861616		
WL 0h	GRF7	0.107695	1.138384		
WL 1h	GRF7	0.001686	0.017817	0.018539	0.002039
WL 1h	GRF7	0.001604	0.01696		
WL 1h	GRF7	0.001972	0.020842		
WL 3h	GRF7	0.000719	0.007602	0.007683	0.00046
WL 3h	GRF7	0.000774	0.008177		
WL 3h	GRF7	0.000688	0.007268		
WL 6h	GRF7	0.000476	0.005036	0.004833	0.000589
WL 6h	GRF7	0.000501	0.005294		
WL 6h	GRF7	0.000394	0.004169		
WL 12h	GRF7	0.000456	0.004818	0.00553	0.000617
WL 12h	GRF7	0.000559	0.005905		
WL 12h	GRF7	0.000555	0.005867		
WL 24h	GRF7	0.000364	0.003844	0.005005	0.001006
WL 24h	GRF7	0.000532	0.005625		
WL 24h	GRF7	0.000525	0.005546		
Biological Repeat 2					
WL 0h	GRF7	0.163684	1.093673	1	0.082729
WL 0h	GRF7	0.140227	0.936941		
WL 0h	GRF7	0.145082	0.969386		
WL 1h	GRF7	0.003598	0.024042	0.023385	0.0006219
WL 1h	GRF7	0.003413	0.022805		
WL 1h	GRF7	0.003489	0.02331		

WL 3h	GRF7	0.001889	0.012619	0.009938	0.004613
WL 3h	GRF7	0.001883	0.012585		
WL 3h	GRF7	0.00069	0.004612	0.004313	0.0010591
WL 6h	GRF7	0.000777	0.005191		
WL 6h	GRF7	0.000469	0.003137		
WL 6h	GRF7	0.000313	0.002088	0.002511	0.0007235
WL 12h	GRF7	0.000501	0.003346		
WL 12h	GRF7	0.000314	0.002098		
WL 12h	GRF7	0.000336	0.002242	0.001834	0.0005513
WL 24h	GRF7	0.000181	0.001207		
WL 24h	GRF7	0.000307	0.0020531		
Biological Repeat 3					
WL 0h	GRF7	0.125288	1.01294	1	0.030432
WL 0h	GRF7	0.132816	1.073804		
WL 0h	GRF7	0.112958	0.913256		
WL 1h	GRF7	0.001711	0.01383	1	0.081053
WL 1h	GRF7	0.002023	0.016355		
WL 1h	GRF7	0.001483	0.011993		
WL 3h	GRF7	0.001806	0.014604	0.01373	0.001492
WL 3h	GRF7	0.001803	0.014579		
WL 3h	GRF7	0.001485	0.012007		
WL 6h	GRF7	0.000324	0.002622	0.003904	0.001127
WL 6h	GRF7	0.000586	0.00474		
WL 6h	GRF7	0.000538	0.004349		
WL 12h	GRF7	2.7E-06	2.19E-05	2.5E-05	4.4E-06
WL 12h	GRF7	3.47E-06	2.81E-05		
WL 12h	GRF7	#VALUE!	#VALUE!		
WL 24h	GRF7	0.00058	0.00469	0.003668	0.001149
WL 24h	GRF7	0.000481	0.00389		
WL 24h	GRF7	0.0003	0.002424		



Sample Name	Target Name	2^-dCt	Normalized	AVG	STD
Biological repeat 1					
WL 0h	At4g09460	0.1596863	1.1277221	1	0.1267545
WL 0h	At4g09460	0.1413235	0.9980421		
WL 0h	At4g09460	0.1237924	0.8742358		
WL 1h	At4g09460	0.0189668	0.1339456	0.1297603	0.0104855
WL 1h	At4g09460	0.0194711	0.1375068		
WL 1h	At4g09460	0.0166846	0.1178286		
WL 3h	At4g09460	0.0249204	0.1759909	0.1625485	0.0117561
WL 3h	At4g09460	0.0222971	0.1574649		
WL 3h	At4g09460	0.0218334	0.1541898		
WL 6h	At4g09460	0.0423551	0.2991166	0.3269815	0.0249842
WL 6h	At4g09460	0.0473574	0.3344431		
WL 6h	At4g09460	0.0491899	0.3473847		
WL 12h	At4g09460	0.0553508	0.3908937	0.3925717	0.0184954
WL 12h	At4g09460	0.0530964	0.3749725		
WL 12h	At4g09460	0.0583181	0.4118488		
WL 24h	At4g09460	0.0420554	0.2970002	0.2428415	0.0696391
WL 24h	At4g09460	0.0378411	0.2672378		
WL 24h	At4g09460	0.0232631	0.1642867		
Biological repeat 2					
WL 0h	At4g09460	0.04498	1.033731	1	0.187908
WL 0h	At4g09460	0.050856	1.168757		
WL 0h	At4g09460	0.034702	0.797511		
WL 1h	At4g09460	0.012726	0.340606	0.3983487	0.100013
WL 1h	At4g09460	0.014821	0.340606		
WL 1h	At4g09460	0.022358	0.513833		

WL 3h	At4g09460	0.014171	0.325674	0.3220095	0.005182
WL 3h	At4g09460	0.013852	0.318345		
WL 3h	At4g09460	#VALUE!	#VALUE!		
WL 6h	At4g09460	#VALUE!	#VALUE!	0.5587222	0.082063
WL 6h	At4g09460	0.021787	0.500695		
WL 6h	At4g09460	0.026836	0.61675		
WL 12h	At4g09460	0.022294	0.51236	0.5401704	0.024094
WL 12h	At4g09460	0.02408	0.55341		
WL 12h	At4g09460	0.024138	0.554741		
WL 24h	At4g09460	0.028623	0.6578	0.7097887	0.073523
WL 24h	At4g09460	0.033147	0.761778		
WL 24h	At4g09460	#VALUE!	#VALUE!		
Biological repeat3					
WL 0h	At4g09460	3.9E-07	1.057109	1	0.065868
WL 0h	At4g09460	3.42E-07	0.927942		
WL 0h	At4g09460	3.74E-07	1.014949		
WL 1h	At4g09460	2.12E-07	0.574553	0.571805	0.015888
WL 1h	At4g09460	2.05E-07	0.554722		
WL 1h	At4g09460	2.16E-07	0.58614		
WL 3h	At4g09460	1.59E-07	0.431783	0.49868	0.061793
WL 3h	At4g09460	2.04E-07	0.553624		
WL 3h	At4g09460	1.88E-07	0.510632		
WL 6h	At4g09460	2.71E-07	0.734103	0.66319	0.066388
WL 6h	At4g09460	2.41E-07	0.652951		
WL 6h	At4g09460	2.22E-07	0.602517		
WL 12h	At4g09460	1.57E-07	0.426092	0.384322	0.04107
WL 12h	At4g09460	1.41E-07	0.382884		
WL 12h	At4g09460	1.27E-07	0.34399		
WL 24h	At4g09460	1.19E-07	0.322418	0.458575	0.124267
WL 24h	At4g09460	1.8E-07	0.487435		
WL 24h	At4g09460	2.09E-07	0.565872		

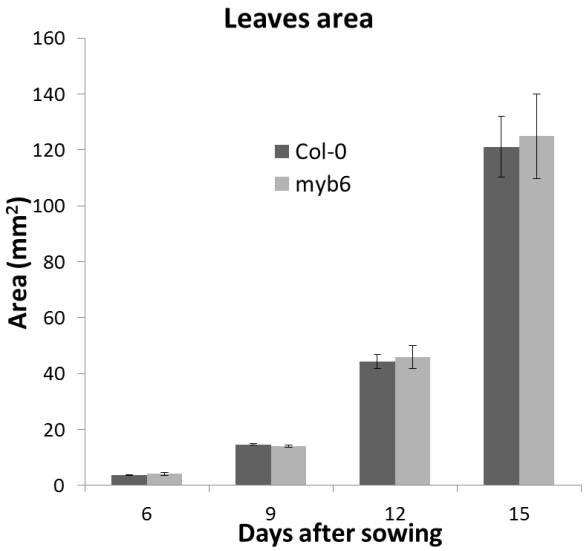


Sample Name	Target Name	2^-dCt	Normalized	AVG	STD
Biological repeat 1					
WL 0h	At5g05800	0.001636	1.06005	1	0.123955
WL 0h	At5g05800	0.001323	0.857457		
WL 0h	At5g05800	0.001671	1.082493		
WL 1h	At5g05800	0.000509	0.33002	0.324357	0.025796
WL 1h	At5g05800	0.000457	0.2962		
WL 1h	At5g05800	0.000535	0.346852		
WL 3h	At5g05800	0.001382	0.895089	0.760522	0.116568
WL 3h	At5g05800	0.001066	0.690633		
WL 3h	At5g05800	0.001074	0.695844		
WL 6h	At5g05800	0.001129	0.731546	0.822069	0.08373
WL 6h	At5g05800	0.001293	0.837919		
WL 6h	At5g05800	0.001384	0.896741		
WL 12h	At5g05800	0.001135	0.735438	0.779947	0.094491
WL 12h	At5g05800	0.001105	0.71593		
WL 12h	At5g05800	0.001371	0.888473		
WL 24h	At5g05800	0.00203	1.315204	1.275589	0.039164
WL 24h	At5g05800	0.001967	1.274672		
WL 24h	At5g05800	0.001909	1.236893		
Biological repeat 2					
WL 0h	At5g05800	0.000834	0.874155	1	0.109002
WL 0h	At5g05800	0.001013	1.061023		
WL 0h	At5g05800	0.001016	1.064823		
WL 1h	At5g05800	0.000657	0.688659	0.636416	0.045957
WL 1h	At5g05800	0.00059	0.618361		
WL 1h	At5g05800	0.000575	0.602229		

WL 3h	At5g05800	0.00122	1.278932	1.25868	0.06825
WL 3h	At5g05800	0.001129	1.182597		
WL 3h	At5g05800	0.001254	1.314512		
WL 6h	At5g05800	0.001132	1.185791	1.373154	0.193402
WL 6h	At5g05800	0.001299	1.361595		
WL 6h	At5g05800	0.0015	1.572077		
WL 12h	At5g05800	0.002202	2.307256	2.163625	0.130885
WL 12h	At5g05800	0.001957	2.051086		
WL 12h	At5g05800	0.002035	2.132534		
WL 24h	At5g05800	0.002211	2.316511	2.215854	0.192057
WL 24h	At5g05800	0.00223	2.336661		
WL 24h	At5g05800	0.001903	1.994391		
Biological repeat 3					
WL 0h	At1g05800	0.000993	0.958068	1	0.030696
WL 0h	At1g05800	0.001048	1.011236		
WL 0h	At1g05800	0.001068	1.030696		
WL 1h	At1g05800	0.000381	0.36745	0.39184	0.019626
WL 1h	At1g05800	0.000431	0.415508		
WL 1h	At1g05800	0.000407	0.392562		
WL 3h	At1g05800	0.000806	0.777975	0.702016	0.054079
WL 3h	At1g05800	0.000696	0.671746		
WL 3h	At1g05800	0.00068	0.656325		
WL 6h	At1g05800	0.000536	0.517477	0.545837	0.039714
WL 6h	At1g05800	0.000624	0.602		
WL 6h	At1g05800	0.000537	0.518034		
WL 12h	At1g05800	0.000708	0.683554	0.721612	0.034616
WL 12h	At1g05800	0.000795	0.767308		
WL 12h	At1g05800	0.00074	0.713973		
WL 24h	At1g05800	0.000675	0.651338	0.702598	0.036414
WL 24h	At1g05800	0.00075	0.723954		
WL 24h	At1g05800	0.000759	0.732501		

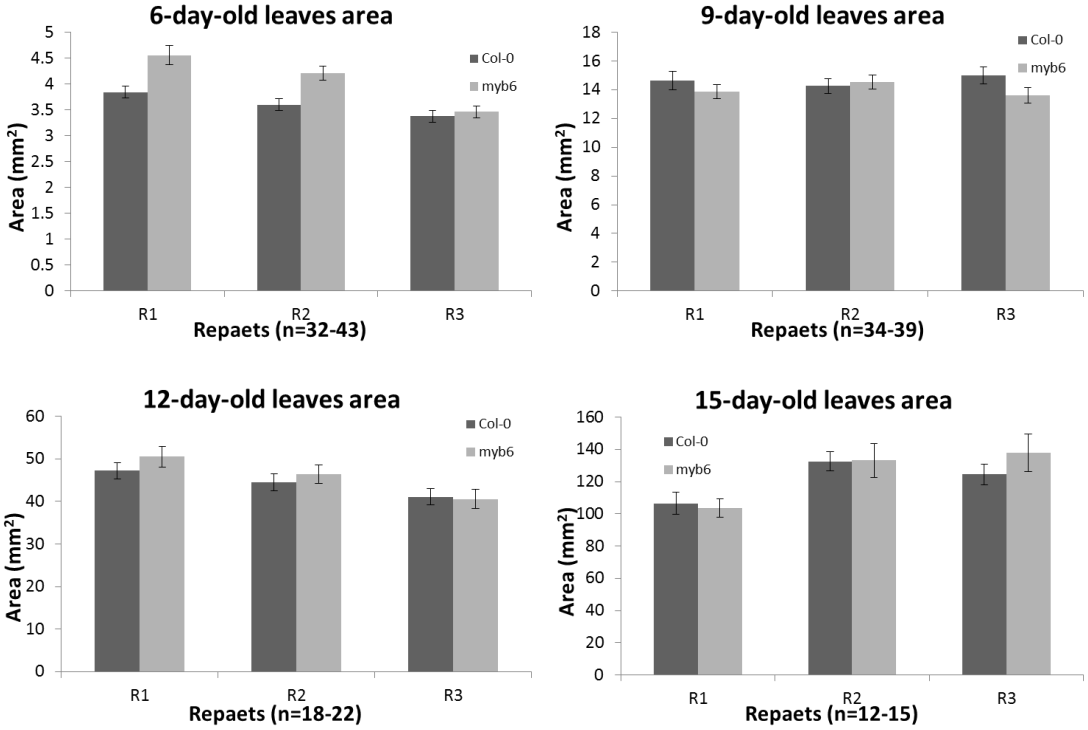
Leaves area measurement over *myb6* mutant

A.



(Overview of leaves area of *myb6* in different stage. X-axis stands for how many days after sowing. Y-axis stands for the total leaves area of a plant. Results are measured with ImageJ and from three independent biological repeats. N is not smaller than 24 in each biological repeats.)

B.



(The details of A. Three biological repeats are shown. “n” means the number of the plants measured per repeat.

The results of genotyping

1. The result of genotyping for SALK_064046 and SALK_064047

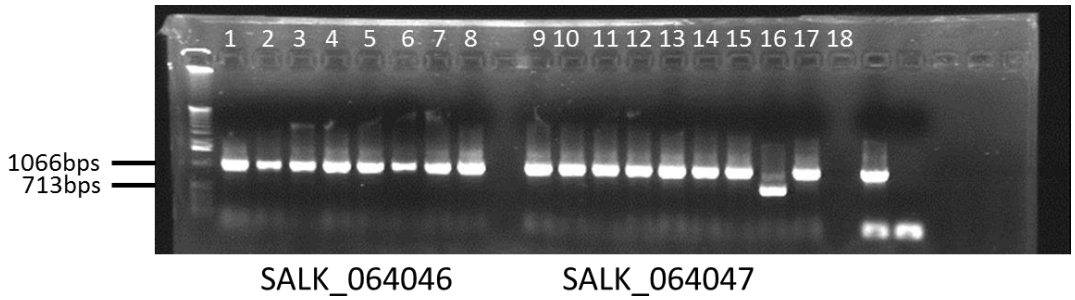


Figure. SALK_064046 and SALK_064047 are the miR396a mutants.The upper bands(1066bp) is from Left Primer and Right Primer, which means that there is no T-DNA insert. The under bands(713bp) is from LBb1.3 and Right Primer, which means that there is a T-DNA insert. #16 of SALK_064047 is selected as homozygosity. And we will use #16 seedlings to observe the phenotypes. While there is no miR858 mutants.

2. The result of genotyping for SALK_074789C

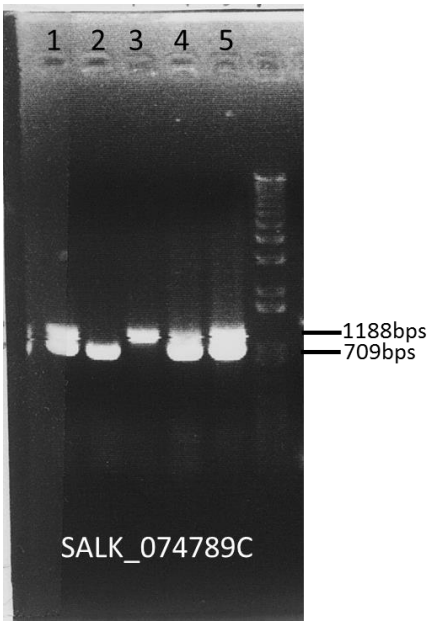


Figure. SALK_074789C is the mutant of *At4g09460*, which is selected miR858 targets.The upper bands(1188bp) is from Left Primer and Right Primer, which means that there is no T-DNA insert. The under bands(709bp) is from LBb1.3 and Right Primer, which means that there is a T-DNA insert. #2 of SALK_074789C is selected as homozygosity. And we will use #2 seedlings to observe the phenotypes. While there is no mutant of selected miR396 targets.

Results of Building and Observing miRNA Overexpression and Target Mimicry Lines

1. The result of colony PCR (*Agro*)for *MIR396B* and *MIR858A* overexpression line

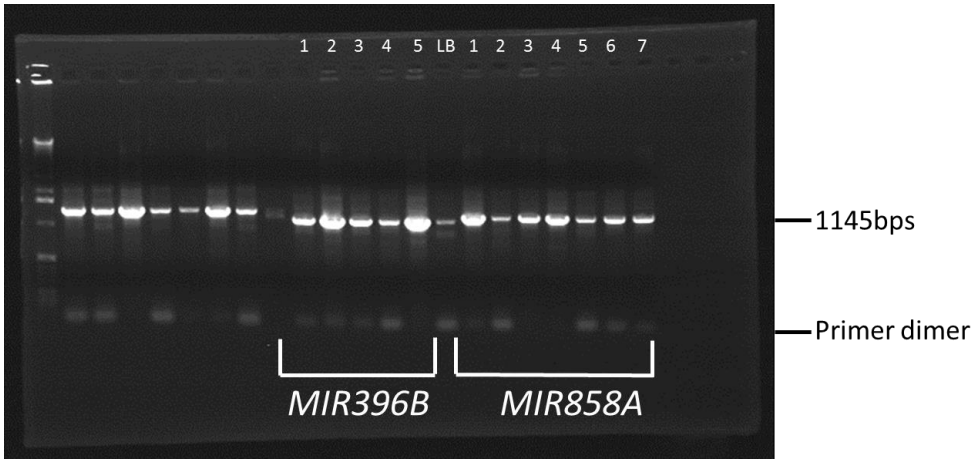


Figure. The band (1145bp) is from 35S-5'-seq and Nos terminator. Because of different enzyme-cut sites (FW:*Sal*I / RV:*Sac*I), the success rate of insertion is very high. Primer dimer form since the used primers may are complementary. #5 of miR396 and #1 of miR858 will be used to conduct dipping on account of the strongest lightness. LB is used as negative control.

2. The result of Southern blotting over T1 transgenic *MIR396B*/ *MIR858A* target mimcry lines.

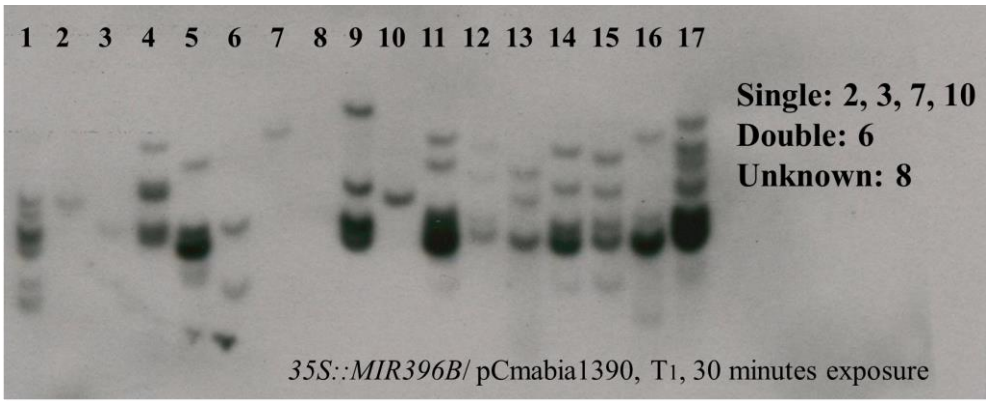


Figure. The imagine of electrophoresis gel of Southern blotting over T1 transgenic *MIR396B* overexpression lines. E*co*RV is used as the enzyme in digestion. The DIG probe is used to mark the number of the inserts. The plants with several inserts are kept, and we will focus on the plants with single and double inserts (#2, 3, 6, 7, 10). *35S::MIR396B*/ pCambia1390 is the construct transformed into *Arabidopsis thaliana.*

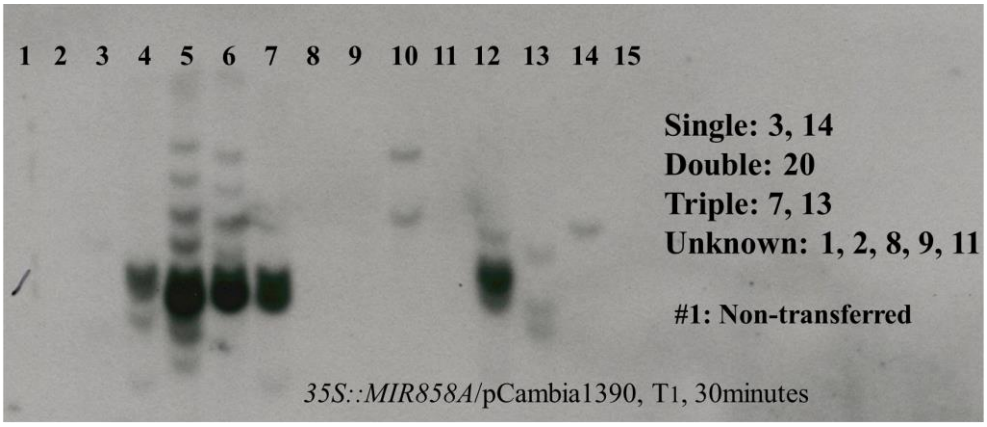


Figure. The imagine of electrophoresis gel of Southern blotting over T1 transgenic *MIR858A* overexpression lines. E*co*RV is used as the enzyme in digestion. The DIG probe is used to mark the number of the inserts. The plants with several inserts are kept, and we will focus on the plants with single, double and triple inserts (#3, 7, 13, 14 ,20). *35S::MIR858B*/ pCambia1390 is the construct transformed into *Arabidopsis thaliana*.

【評語】060011

1. 報告內容十分混亂，有些地方也不對。

2. 必須再強調 miRNA 在調整的創新性或你們的領先性，或為何 miRNA 對快速反應特別重要，否則只是眾多調控機制的一個。

3. 如此多的基因調控，例如有眾多基因可調控照光後花青素的累積，如何呈現你們所選定的重要性，需再加強。