Abstract

The evolutionary conserved WD-40 protein PRL1 plays important roles in immunity and development. Here we show that PRL1 is required for the accumulation of microRNAs (miRNAs) and small interfering RNAs (siRNAs). PRL1 positively influences the processing of miRNA primary transcripts (pri-miRNAs) and double-stranded RNAs (dsRNAs). Furthermore, PRL1 interacts with the pri-miRNA processor, DCL1, and the dsRNA processors (DCL3 and DCL4). These results suggest that PRL1 may function as a general factor to promote the production of miRNAs and siRNAs. We also show that PRL1 is an RNA-binding protein and associates with pri-miRNAs in vivo. In addition, prl1 reduces pri-miRNA levels without affecting pri-miRNA transcription. These results suggest that PRL1 may stabilize pri-miRNAs and function as a co-factor to enhance DCL1 activity. We further reveal the genetic interaction of PRL1 with CDC5, which interacts with PRL1 and regulates transcription and processing of pri-miRNAs. Both miRNA and pri-miRNA levels are lower in cdc5 prl1 than those in either cdc5 or prl1. However, the processing efficiency of pri-miRNAs in cdc5 prl1 is similar to that in cdc5 and slightly lower than that in prl1. Based on these results, we propose that CDC5 and PRL1 cooperatively regulate pri-miRNA levels, which results in their synergistic effects on miRNA accumulation, while they function together as a complex to enhance DCL1 activity.

Introduction

In plants and animals, microRNAs (miRNAs), ~20–25 nucleotides (nt) in size, regulate gene expression in various biological processes such as development and metabolism [1–3]. They are produced as duplexes through precise excision from imperfect fold-back primary transcripts (pri-miRNAs) [1–3]. In the miRNA duplex, the miRNA strand is loaded into ARGONAUTE (AGO) proteins to repress the expression of target genes containing its complementary sequences while the other strand (passenger strand; miRNA*) is degraded [1–3]. Plants and animals also use small interfering RNAs (siRNAs) to repress gene expression. siRNAs are chemically identical to miRNAs [2]. However, they are produced from long double stranded RNAs. The two major classes of plant siRNAs are siRNAs derived from repeated DNAs (rasiRNAs) and trans-acting siRNAs (ta-siRNAs) [4,5].

In plants, most pri-miRNAs are transcribed by DNA-dependent RNA polymerase II (Pol II) from endogenous miRNA encoding genes [MIIR] [1,2]. The mediator complex and Negative on TATA less2 (NOT2; a transcription factor) regulate the transcription of MIIR [6,7]. After generation, pri-miRNAs are proposed to be stabilized by DAWDLE (DDL), an RNA binding protein [8]. Pri-miRNAs are then processed to stem-loop precursors (pre-miRNAs) and finally to the miRNA/miRNA* duplex by Dicer-LIKE 1 (DCL1; an RNAse III enzyme) in the nucleus in plants [9,10]. The C2H2 zinc-finger protein SERRATE (SE) and the RNA binding proteins HYPONASTIC LEAVES 1 (HYL1) and TOUGH (TGH) form a complex with DCL1 to ensure efficient and accurate process of pri-miRNAs [9,11–17]. To ensure its proper function, HYL1 needs to be dephosphorylated during pri-miRNA processing [18]. Several other proteins including DDL, Cap-Binding Protein 20 (CBP20), CBP80, RACK1 and NOT2 are associated with the DCL1 complex to facilitate miRNA maturation [7,8,19–21]. NOT2 and MODIFIER OF SNC1, 2 (MOS2; an RNA binding protein) have been shown to guide the correct localization of the DCL1 complex [7,22]. SICKLE (SIC; a prolactin rich protein) is shown to regulate the accumulation of some miRNAs [23]. Besides protein factors, the structure of pri-miRNAs plays essential roles in regulating DCL1 activity [24–27]. For instance, an imperfectly paired lower stem of ~15 bp below the miRNA:mRNA* duplex is crucial for the initial pri-miRNA cleavage [25–27].

We previously showed that Cell Division Cycle 5 (CDC5), a DNA-binding protein, positively regulates miRNA biogenesis in Arabidopsis [28]. CDC5 interacts with Pol II and MIIR promoters [28]. Lack of CDC5 reduces the occupancy of Pol II at MIIR promoters and pri-miRNA levels, suggesting that CDC5 is a positive transcription factor of MIIR [28]. Besides acting as a transcription factor, CDC5 functions as a co-factor of the DCL1 complex to participate pri-miRNA processing [28]. CDC5 is a
Author Summary

PRL1, a conserved WD-40 protein, is required for plant development and immune responses. However, its functional mechanisms are not well understood. Here, we show the positive impact of PRL1 on the accumulation of miRNAs and siRNAs, which are key regulators of plant growth and immunity. PRL1 interacts with multiple DCLs (the processors of miRNAs and siRNAs) and is required for their optimal activities, suggesting that PRL1 acts as a general factor to facilitate the production of miRNAs and siRNAs. In addition, PRL1 is an RNA-binding protein, binds pri-miRNAs in vivo and positively influences the levels of pri-miRNAs without affecting the promoter activities of genes encoding pri-miRNAs. These results suggest that PRL1 may also stabilize pri-miRNAs. We further show that PRL1 and its interactor CDC5 (a DNA-binding protein) synergistically regulate pri-miRNA levels, resulting in enhanced effects on miRNA accumulation, although they function together as a complex to facilitate DCL1 activity.

Results

The accumulation of miRNAs and siRNAs is reduced in prl1-2

Given the role of CDC5 in miRNA biogenesis, it is possible that other components of the MAC complex may also be required for miRNA accumulation. Therefore, we examined the effect of the mutants mac3b (SALK_050811), mos4 (SALK_009085C1G) and prl1-2 on miRNA abundance using Northern blot. We also included sncl (SALK_047058C) in the analysis since SNC1 is related to the MAC complex and sncl causes development defects. These mutants are likely null since the transcripts of corresponding genes could not be detected by RT-PCR (Figure S1A). Like in cdc5-1, the abundance of all four tested miRNAs (mir167, mir171, mir172 and mir173) was decreased in prl1-2 compared to Col (wild-type control). In contrast, miRNA levels in mos4, mac3b and sncl were comparable with those in Col (Fig. 1A). We examined the accumulation of additional miRNAs in prl1-2 and found that all these miRNAs were reduced in abundance in prl1-2 relative to Col (Fig. 1B). In addition, expression a wild-type copy of PRL1 fused with a YFP tag under the control of its native promoter (pPRL1::PRL1-YFP) fully recovered miRNA levels in prl1-2 (Fig. 1B). These results demonstrated that PRL1 but not MOS4 and MAC3b is required for miRNA accumulation. We next analyzed the transcript levels of several miRNA targets (ARF3, CUC1, MYB33, MYB65 and PHV) in prl1-2 and Col by quantitative RT-PCR (qRT-PCR) in order to test the effect of prl1-2 on miRNA function. The transcription levels of these targets were slightly increased in prl1-2 relative to Col (Figure. S1B). The PRL1 transgene fully recovered miRNA function in prl1 (Figure S1B). We also asked if PRL1 has a role in siRNA biogenesis. The levels of nine examined siRNAs (three ta-siRNAs and six ra-siRNAs) were reduced compared to those in Col (Fig. 1B and 1C), which was complemented by the expression of pPRL1::PRL1-YFP. These results revealed that like CDC5, PRL1 positively regulates the levels of miRNAs and siRNAs in Arabidopsis.

PRL1 associates with Pol II and DCL1

The PRL1-CDC5 interaction suggests that similar to CDC5, PRL1 may act as a co-factor of Pol II and DCL1 to regulate miRNA accumulation. To test these two possibilities, we first examined the PRL1-Pol II association using co-immunoprecipitation (co-IP) assay. In this experiment, anti-YFP and anti-RPB2 that detects the second largest subunit of Pol II (RPB2) [6] were used to capture the PRL1-YFP and Pol II complex, respectively, from the protein extracts of prl1-2 complementation line expressing the pPRL1::PRL1-YFP transgene. After IP, PRL1-YFP was detected in the Pol II precipitates whereas RPB2 existed in the PRL1-YFP complex (Fig. 2A and 2B). In contrast, no interaction was detected in the control reactions (Fig. 2A and 2B), demonstrating the PRL1-Pol II association.

We next tested the association of PRL1 with the components of DCL1 complex using a bimolecular fluorescence complementation (BiFC). In the BiFC assay, transient co-expression of PRL1 fused with C-terminal fragment of cyan fluorescent protein (cCFP) with DCL1, SE, HYL1 or CDC5 fused with the N-terminal fragment of Venus (nVenus) produced yellow fluorescence signals (Fig. 2C), suggesting that PRL1 might associate with the DCL1 complex. To verify this result, we tested co-IP of PRL1 with DCL1 and SE. After PRL1-YFP or YFP was transiently co-expressed with DCL1-MYC and SE-MYC fusion proteins in N. benthamiana, respectively, IP was performed with anti-YFP or anti-MYC antibodies. Western blots detected PRL1-YFP in the DCL1-MYC and SE-MYC complexes and DCL1-MYC/SE-MYC in the PRL1-YFP precipitates, suggesting that PRL1-YFP and DCL1/SE reciprocally pulled down each other (Fig. 2D, 2E, 2F and 2G). As a control, YFP did not show interaction with either DCL1 or SE. These results demonstrated the association of PRL1 with DCL1 and SE.

PRL1 positively influences pri-miRNA levels without affecting MIR promoter activity

The interaction of PRL1 with Pol II suggests that PRL1 may positively regulate MIR transcription. If so, lack of PRL1 will...
PRL1 and Small RNA Biogenesis

Figure 1. PRL1 is required for the accumulation of miRNAs. (A) The effect of various MAC components on the abundance of miRNAs (B) The levels of miRNAs are reduced in prl1-2. (C) The levels of siRNAs are reduced in prl1-2. Col: wild-type control. For mir159/319: upper band, mir159; lower band, mir319. Northern blot was used to detect small RNAs and the radioactive signals were quantified with ImageQuant (V5.2). To determine the amount of miRNAs/siRNAs in various mutants relative to that in Col, the radioactive signals of miRNAs/siRNAs were normalized to U6 RNA and compared with that in Col (set as 1). The numbers indicate the average value of three repeats (P<0.05).

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The role of PRL1 in siRNA biogenesis

We next asked the role of PRL1 in siRNA biogenesis, as prl1-2 reduces the accumulation of siRNAs. By analog, we examined the interaction of PRL1 with DCL3 and DCL4 and the effect of prl1-2 on dsRNA processing. To test the PRL1-DCL3/DCL4 interaction, we expressed a recombined PRL1 fused with a maltose-binding protein at its N-terminus (MBP-PRL1) and MBP in E. coli. The protein extracts containing MBP-PRL1 or MBP were mixed with protein extracts containing DCL3-YFP or DCL4-YFP, which were transiently expressed in N. benthamiana. Then the DCL3-YFP or DCL4-YFP complex was IPed with anti-YFP antibodies. MBP-PRL1, but not MBP, was co-IPed with DCL3-YFP and DCL4-YFP (Fig. 5A). In addition, YFP did not interact with MBP or MBP-PRL1. These results demonstrated that PRL1 interacts with DCL3 and DCL4 (Fig. 5A).

To test the effect of prl1 on dsRNA processing, we generated ~460 bp dsRNAs through in vitro transcription of a DNA fragment (5’ portion of UBIQUITIN 5) containing the T7 promoter at the end of each strand under the presence [α-32P] UTP. The radioactive labeled dsRNA then incubated with prl1 or Col protein extracts. The production of both 21 nt and 24 nt small RNAs was impaired in prl1 compared with that in Col and that in the complementation line (Fig. 5B). This result indicated that multiple DCL activities are impaired by prl1-2, because DCL3 is responsible for the production of 24 nt small RNAs and DCL1/DCL4 is involved in the production of 21 nt small RNAs from dsRNAs.

PRL1 and CDC5 synergistically regulate miRNA accumulation

CDC5 and PRL1 have been shown to directly interact with each other. Both CDC5 and PRL1 interact with DCL1 and positively impair MIR transcription, resulting in reduced levels of pri-miRNAs. To test this, we compared the pri-miRNA levels in prl1-2 with those in Col using qRT-PCR. In fact, the levels of all seven examined pri-miRNAs in prl1-2 were less than those in Col (Fig. 3A), which were recovered in the complementation line of prl1-2 (Fig. 3A). To test whether the reduction of pri-miRNA levels is due to impaired MIR promoter activity, we introduced the pri-1-2 mutation into a Col transgenic line containing a single cope promoter (Fig. 3B). To test the role of pri-miRNAs in the protein extracts of young flower buds of prl1-2, we did not show obvious effect on MIR172b promoter activity (Figure S2A).

Next we evaluated the effect of prl1-2 on the half-lives of pri-miRNAs using cordycepin as a transcriptional inhibitor [30]. Two-week old plants were transferred to medium containing cordycepin. After incubation was stopped at various time points, we measured the levels of pri-miR164a and pri-miR167a using qRT-PCR. The results showed that the degradation rate of pri-miR164a and pri-miR167a in prl1-2 is similar to that in Col (Figure S2B).

PRL1 functions in miRNA maturation

We next asked whether PRL1 has a role in processing of miRNA precursors through an in vitro processing assay [13,31] since it is associated with DCL1 and SE. In this experiment, a portion of pri-miRNA that contains the stem-loop of miRNA with 6-nt arms at each end (MIR162b; Fig. 4A) and pre-miRNA162b (Fig. 4B) were first produced through in vitro transcription in the presence of [α-32P] UTP. [α-32P] labeled MIR162b and pre-miRNA162b were then processed in the protein extracts of young flower buds of prl1-2 or Col. The production of miR162b from both MIR162b and pre-miRNA162b in prl1-2 at various time points was less than that in Col (Fig. 4C and 4D). The processing of MIR162b and pre-miRNA162b was recovered in the PRL1 complementation line (Figure S3) The levels of miR162 produced from MIR162b and pre-miRNA162b in prl1 at 80 min were ~40% of those produced in Col (Fig. 4E and 4F). These results suggested that PRL1 might have a role in promoting miRNA maturation.
regulate miRNA processing. These results raise a possibility that CDC5 and PRL1 may act as a complex to regulate DCL1 activity. In addition, CDC5 regulates MIR promoter activity while PRL1 does not. These suggest that PRL1 and CDC5 might act additionally in miRNA pathway. To test these two possibilities, we constructed a cdc5-1 prl1-2 double mutant by crossing prl1-2 into cdc5-1 and compared miRNA levels in cdc5-1 prl1-2 with those in cdc5-1 and prl1-2, respectively. The cdc5-1 prl1-2 double mutant displayed more severe developmental defects than either cdc5-1 or prl1-2, suggesting that PRL1 and CDC5 function additionally in regulating development (Fig. 6A). Northern blot analyses showed that the levels of several examined miRNAs in cdc5-1 prl1-2 were lower than those in either prl1-2 or cdc5-1 (Fig. 6B), indicating that PRL1 and CDC5 function synergetically in miRNA pathway.

There are at least two possible explanations for the further reduction of miRNA levels in cdc5-1 prl1-2 relative to either cdc5-1 or prl1-2.
Figure 3. PRL1 positively influences pri-miRNA levels. (A) The abundance of pri-miRNAs in inflorescences of prl1-2 and Col. The transcript levels of pri-miRNAs in prl1-2 were determined by quantitative RT-PCR (qRT-PCR), normalized to that of UBQUITIN 5 (UBQ5) and compared with those in Col. Value of Col was set to 1. Error bars indicate standard deviation of three technical replications. **: P < 0.01. (B) GUS expression in PRL1+/prl1-2 harboring pMir167a::GUS. PRL1+/prl1-2 harboring pMir167a::GUS. GUS transcript levels were determined by qRT-PCR and normalized to UBQ5. Value of PRL1+ was set to 1. Standard deviation of three technical replications was shown as error bars. P = 0.11 (t-test), doi:10.1371/journal.pgen.1004841.g003

1 or prl1-2 based on the fact that both PRL1 and CDC5 positively regulate pri-miRNA levels and miRNA maturation. One is that pri-miRNA levels might be further reduced in cdc5-1 prl1-2. The other is that the processing efficiency of miRNA precursors might be lower than either cdc5-1 or prl1-2. To test these two possibilities, we first determined the pri-miRNA levels in cdc5-1 prl1-2, cdc5-1 and prl1-2 through qRT-PCR. The levels of several tested pri-miRNAs were decreased in cdc5-1 prl1-2 when compared with those in either cdc5-1 or prl1-2 (Fig. 6C), suggesting that CDC5 and PRL1 indeed act synergistically in regulating pri-miRNA levels. Next, we evaluated the in vitro processing of pre-miR162b in cdc5-1 prl1-2. The amount of miR162b produced in cdc5-1 prl1-2 was similar to that in cdc5-1 and slightly lower than that in prl1-2 (Fig. 6D), suggesting that PRL1 and CDC5 may not act additionally in promoting miRNA maturation.

PRL1 binds pri-miRNAs in vitro and in vivo

Given the role of PRL1 in RNA metabolism, it is reasonable to speculate that PRL1 might have an RNA-binding activity. We therefore performed an in vitro RNA pull-down assay to test this possibility. In this assay, recombinant PRL1 fused with a maltose-binding protein at its N-terminus (MBP-PRL1) and MBP were expressed in E.coli and purified with amylose resin (Fig 7A). MBP-PRL1 and MBP were then incubated with [32P]-labeled MIR162b or pre-miR162b, respectively. MBP-PRL1 but not MBP bound MIR162b and pre-miR162b after incubation. In addition, when excess amount of unlabeled MIR162b or pre-miR162b was added in the reaction, radioactive labeled MIR162b or pre-miR162b could not be retained in the MBP-PRL1 complex. These results suggested that PRL1 binds RNAs in vitro. We next tested the binding ability of MBP-PRL1 to dsRNA, ssRNA and DNA using the in vitro RNA pull-down assay described above. MBP-PRL1 was able to bind a ~100-nt RNA corresponding to a portion of the 5’ end of the UBQUITIN 5 (UBQ5) [13], but not an in vitro synthesized ~50 bp DNA fragment [32] and a dsRNA generated through in vitro transcription vitro transcription of a DNA fragment (5’ portion of UBQ5, ~460 bp) containing the T7 promoter at end of each strand [13] (Fig. 7B).

Next, we performed an RNA immunoprecipitation (RIP) assay to test whether PRL1 binds pri-miRNAs in vivo [13]. Scellings of the prl1-2 complementation line expressing pPRL1::PRL1-YFP transgene and the control plants harboring YFP were used for RIP. After PRL1-YFP or YFP complex were precipitated with anti-YFP antibody, pri-miRNAs were detected with RT-PCR. Several tested pri-miRNAs (pri-miR159a, pri-miR167a, pri-miR171 and pri-miR172a) existed in the PRL1-YFP complex but not in the YFP complex and no Anti-body (NoAb) controls (Fig. 7C). These results suggested that PRL1 associates with pri-miRNAs in vivo.

Discussion

In this study, we identify PRL1, a WD-40 protein, as an important regulator of miRNA accumulation. Several evidences including reduced accumulation of pri-miRNAs and miRNAs in prl1, PRL1-DCL1 interaction and PRL1-pri-miRNA association demonstrate that PRL1 positively impacts miRNA biogenesis. It has been suggested that PRL1 influences plant immunity and development through its impacts on RNA processing [29,33]. Given the essential roles of miRNAs in plant immunity and development, it is possible that reduced miRNA levels in prl1 may partially contribute to the observed phenotypes.

PRL1 likely has a role in promoting miRNA maturation, as lack of PRL1 reduces processing of MIR162b and pre-miR162b. PRL1 interacts with the DCL1 complex and does not positively regulate the transcription of genes involved in miRNA biogenesis (Figure S4), suggesting that PRL1 may act as a co-factor to regulate DCL1 activity. CDC5, a direct interactor of PRL1 also regulates the DCL1 activity through its interaction with the helicase and dsRNA binding domains of DCL1. The effect of PRL1 on pri-miRNA processing appears to be weaker than that of CDC5. The processing efficiency of MIR162b and pre-miR162b in cdc5-1 prl1-2 is similar to that in cdc5-1 and slightly lower than that in prl1-2. This result suggests that PRL1 and CDC5 may act together as a complex to regulate DCL1 activity. Furthermore, gel filtration analysis suggests that PRL1 may not affect DCL1-CDC5 association (Figure S5). Thus, it is possible that PRL1 may act as accessory factor to facilitate CDC5 function. PRL1 also positively regulates the pri-miRNA levels since prl1 reduces the accumulation of pri-miRNAs. We previously showed that CDC5 interacts with Pol II and positively regulate MIR transcription [28]. Since PRL1 associates with Pol II as well, it is possible that PRL1 acts as a component of the CDC5 complex to regulate MIR promoter activity. However this seems not to be the
case, as loss-of-function of PRL1 does not affect the GUS levels driven by the MIR167a promoter. Consistent with this notion, the levels of pri-miRNAs are further reduced in cdc5-1 prl1-2 compared with cdc5-1 or prl1-2. Given the fact that PRL1 binds pri-miRNAs in vitro and vivo, we propose that PRL1 may stabilize pri-miRNAs. Indeed, the fact that the half-life of pri-miR164a and pri-miR167a in prl1 is similar to that in Col suggests that the degradation of pri-miRNAs may be increased in prl1, because less efficient processing may lead to increased abundance of pri-miRNAs in prl1. However, we cannot rule out the possibility that PRL1 acts in MIR transcription after initiation, as it associates with Pol II.

In summary, we reveal that PRL1 positively regulates miRNA levels through its impacts on pri-miRNA levels and processing. PRL1 functions additively with its interactor CDC5 as miRNA abundance is lower in cdc5-1 prl1-2 than in cdc5-1 or prl1-2.

Figure 4. PRL1 is required for miRNA maturation in vitro. (A) and (B) A schematic diagram of the MIR162b (A) and pre-miR162b (B) used in vitro processing assay. (C) and (D) The amount of miR162b produced from MIR162b and pre-miR162b were reduced in prl1-2. Proteins were isolated from inflorescences of prl1-2 and Col and incubated with MIR162b or pre-miR162b. The reactions were stopped at various time points as indicated in the picture. (E) and (F) Quantification of miR162b production in prl1-2 compared to that in Col. Quantification analysis was performed at 80 min. The radioactive signal of miR162 were normalized to input and compared with that of Col. The amount of miR162 produced in Col was set as 1. The value represents mean of three repeats (*** P<0.001; t-test).

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Figure 5. The role of PRL1 in siRNA biogenesis. (A) PRL1 interacts with DCL3 and DCL4. Co-IP was performed to detect the interaction of PRL1 with DCL3 or DCL4. MBP and MBP-PRL1 fused protein were expressed in E.coli. YFP, DCL3-YFP and DCL4-YFP were expressed in N. benthamiana leaves. Anti-YFP was used for IP. For loading, ten percent and one percent of input proteins were used for IP and Co-IP, respectively. (B) prl1-2 impairs siRNA production from double-stranded RNAs (dsRNAs). Protein extracts isolated from inflorescences of Col, prl1-2 and prl1-2 containing a PRL1-YFP transgene were incubated dsRNAs for 120 min. dsRNAs were synthesized through in vitro transcription of a DNA fragment (5’ portion of UBQ5 gene, ~460 bp) under the presence of [α-32P] UTP.

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synergistic effect of CDC5 and PRL1 on miRNA levels can be explained by their different roles in controlling pri-miRNA levels rather than their function in promoting miRNA maturation. Besides CDC5 and PRL1, the core components MAC complex includes MOS4, MAC3A and MAC3B [29]. We show that MOS4 and MAC3B have no impact on miRNA levels. However, whether MAC3B has a role in miRNA accumulation needs to be further explored since it acts redundantly with MAC3A [29]. The MAC complex appears to have a role in siRNA biogenesis. Both CDC5 and PRL1 promote the accumulation of siRNA [28] while MOS4 is required for the accumulation of ra-siRNAs [34]. How does MAC participate in siRNA biogenesis? We have showed both PRL1 and CDC5 interact with the DCL1 complex and regulate its activity. By analogy, it is possible that the MAC complex associates with the DCL3 complex to regulate its activity. In fact, DCL3 interacts with PRL1. prl1 also reduces the abundance of ta-siRNAs, whose production requires DCL4 and DCL1-dependent miRNAs. Since PRL1 interacts with DCL4 and is required for the accumulation of DCL1-dependent miRNAs, it may promote ta-siRNA production through facilitating DCL4 function and miRNA production. The MAC complex is an evolutionarily conserved complex [29]. As many aspects of small RNA pathway are conserved, it is tempting to propose that the counterparts of MAC play some roles in small RNA pathways in other organisms.

Materials and Methods

Plant materials

The macOSb (SALK_050811), mos4 (SALK_0090851C), prl1-2 (Salk_008466), sne1 (SALK_047058C) and cdc5-1 (SAIL_207_F03) mutants were ordered from Arabidopsis Biological Resources Center (ABRC). All of them are in the Columbia-0 genetic background. Transgenic line containing a single copy of pMIR167a::GUS was crossed to prl1-2. In the F2 population, PRL1/PRL1, PRL1/prl1-2 and prl1-2/prl1-2 harboring pMIR167a::GUS were identified through PCR genotyping for prl1-2 and GUS.

Plasmid construction

PRL1 genomic DNA was amplified from Col genome and cloned to pMDC204 binary vector to generate pPRL1::PRL1-YFP construct. The construct was transformed to prl1-2. The full-length PRL1 cDNA was amplified by RT-PCR and ligated to pMAL-c5x (NEB) to generate MBP-PRL1. To generate the cCFP-PRL1 fusion vector, the PRL1 cDNA was first cloned into the pSAT4-cCFP-C vector [35]. The DNA fragment containing cCFP-PRL1 was released by I-SceI restriction enzyme and subsequently cloned into the pPZP-RCS2-ocs-bar vector. All the primers are listed in Table S1.

Co-IP assay

In the PRL1-PoII co-IP experiment, proteins were extracted from the transgenic plants harboring the pPRL1::PRL1-YFP transgene and incubated with anti-YFP (Clontech) or anti-RPB2 antibodies coupled to protein G agarose beads (Clontech) for 4 h at 4°C. After the beads were washed five times with protein extraction buffer, proteins were resolved by SDS/PAGE. Anti-YFP and anti-MYC (MBL) antibodies were used to detect PRL1-YFP/YFP and DCL1-MYC/SE-MYC, respectively.

Figure 6. PRL1 and CDC5 synergistically regulate miRNA accumulation. (A) Morphological phenotypes of Col, cdc5-1, prl1-2 and cdc5-1 prl1-2. (B) The abundance of miRNAs is lower in cdc5-1 prl1-2 than that in cdc5-1 or prl1-2. Small RNAs were detected by Northern Blot. To determine the amount of miRNAs, radioactive signals of miRNAs were normalized to U6 RNA. The number represents the relative abundance compared to Col (set as 1) quantified by three repeats (P<0.05). (C) The abundance of pri-miRNAs is reduced in cdc5-1 prl1-2. The levels of pri-miRNAs in various mutants were determined by qRT-PCR, normalized to UBQUITIN5 (UBQ5) and compared with those of Col (set as 1). Standard deviation of three technical replications was shown as error bars. **: P<0.01. (D) miR162b production from pre-miR162b in Col, cdc5-1 prl1-2, cdc5-1 and prl1-2. The reaction was stopped at 120 min. The radioactive signals of miR162b were normalized to input. The number represents the relative production in various genotypes compared to Col (set as 1) quantified by three repeats (P<0.05). doi:10.1371/journal.pgen.1004841.g006

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DNA analysis

Northern blot was used to detect small RNA abundance as described [13]. qRT-PCR was performed to detect the levels of pri-miRNAs, transcripts of miRNA targets and GUS using cDNA templates reverse transcribed by the SuperScript III (Invitrogen) and oligo dT18 primer. qRT-PCR was run on an iCycler apparatus (Bio-Rad). RNA pull-down were performed according to Ren et al [13]. MBP and MBP-PRL1 were expressed in E.coli. MIR162b, pre-miR162b, dsRNAs and ssRNA were produced by in vitro transcription with T7 RNA polymerase at the presence [α-32P] UTP whereas DNA was synthesized at IDT and labeled with T4 PNK at the presence [γ-32P] ATP. [32P]-labeled probes are incubated with amylase resin beads combined MBP or MBP-PRL1 at 4 °C for 1 hour. After 4 times wash with washing buffer, DNA or RNA are extracted and resolved on PAGE gel. Radioactive signals were detected with a PhosphorImager and quantified by ImageQuant version 5.2. RIP was performed according to [13,36]. Seedlings of transgenic plants harboring the pPRL1::PRL1-YFP transgene or YFP were used to examine the RNA binding activity of PRL1 in vivo. All the primers are listed in Table S1.

RNA half-life assay was performed according to Lidder et al [30]. Two-week-old Col and prl1-2 seedlings were transferred to flask with incubation buffer (1/2 MS medium), respectively. After 30 min incubation, 3′-deoxyadenosine (Cordycepin, Sigma) was added to final concentration of 0.6 mM (time 0). Seedlings were collected at various time points (0, 15, 30, 60, 90, 120 and 240 min). qRT-PCR then was performed to detect the transcript levels of pri-miRNAs and DDL. For quantification, the transcript levels of pri-miRNAs and DDL at various time points were normalized to that of eIF4a respectively. Value of time 0 was set to 1. Error bars indicate standard deviation of three technical replications. Three biological repeats were performed and similar results were obtained.

Gel filtration analysis

The gel filtration was performed on an HPLC system and a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare) at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected every minute. Fractions were separated by 8% SDS-PAGE and analyzed by Western blotting using antibodies recognizing CDC5, CDC6 or YFP. The protein standards (Bio-Rad, http://www.bio-rad.com/) were used to calibrate the column contain five size standards.

Supporting Information

Figure S1 (A) The levels of MIR162b, MIR164a, PRL1 and Small RNA Biogenesis in four null mutants detected by RT-PCR. The T-DNA line of mac3b (SALK_050611), mos4 (SALK_090651C), prl1-2 (SALK_039427), snc1 (SALK_047050C) are all in Columbia-0 genetic background. (B) The transcript levels of several small RNA targets in prl1-2, Col and complementation line. The amount of target transcripts in prl1-2 and complementation line were normalized with UBQUITIN5 (UBQ5) and compared with that of Col (set as 1). Error bars indicate standard deviations of three technical replications. *:P<0.05; **:P<0.01.

Figure S2 (A) The levels of GUS mRNA in pPRL1* and prl1-2 harboring pMIR172a::GUS. GUS mRNA levels were determined by qRT-PCR and normalized to UBQ5. Value of PRL1* was set to 1. Standard deviation of three technical replications was shown as error bars. (B) Pri-miR164a, pri-miR167a and DDL mRNA decay in the half-life assay. Two-week-old Col and prl1-2 seedlings were treated with 0.6 mM 3′-deoxyadenosine (Cordycepin, Sigma) at various times (0, 15, 30, 60, 90, 120 and 240 min). qRT-PCR was performed to detect pri-miRNA, and DDL.
transcription levels and normalized to internal control (eIF4e).
Value of time 0 was set to 1. Error bars indicate standard deviation of three technical replications.

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Figure S3 The PRL1-YFP transgene restores in vitro processing of MIR162b and pre-miR162b in prl-1. Protein extracts isolated from inflorescences of Col, prl-1 and prl-1 containing a PRL1-YFP transgene were incubated with MIR162b and pre-miR162b for 120 min.

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Figure S4 The effects of prl-1 on the expression of several genes involved in miRNA biogenesis. (A) The transcript levels of several genes involved in miRNA biogenesis determined by qRT-PCR. UBO5 was used as a control. Standard deviation of three technical replications was shown as error bar. (B) DCL1, (C) HYL1 and (D) CDC5 protein levels in various genotypes detected by western blot. Controls were dcl-1-9 containing a truncated DCL1 protein, hyl-1 lacking of HYL1 and cdc-5-1 lacking CDC5.

(TIF)

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