MicroRNA840 accelerates leaf senescence by targeting the overlapping 3’UTRs of PPR and WHIRLY3 in Arabidopsis thaliana

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Running title: MiR840 regulates plant senescence

Highlight: MicroRNA840 (miR840) has a unique miRNA-target configuration regulating PPR and WHIRLY3 genes in Arabidopsis. MiR840 is highly expressed at the onset of plant senescent stage. Both PPR and WHIRLY3 transcripts are specifically targeted in vivo within their 3’UTR region by mature miR840 or its star strand in vivo. Interestingly, PPR expression is mainly repressed on mRNA transcript level by cleavage, while WHIRLY3 is predominantly translationally inhibited. We conclude that miR840 enhances plant senescence via post transcriptional gene silencing of PPR and WHIRLY3, which appear to be novel negative joint regulators of plant senescence.

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Abstract

MicroRNAs (miRNAs) negatively regulate gene expression by cleaving the target mRNA and/or impairing its translation, thereby playing a crucial role in plant development and environmental stress responses. In Arabidopsis, MIR840 is located within the overlapping 3’UTR of PPR and WHIRLY3 (WHY3), both being predicted targets of miR840. Gain- and loss-of-function of miR840 in Arabidopsis resulted in opposite senescent phenotypes. Highest expression of pri-miR840 is observed at senescence initiation, and is negatively correlated with a significant reduction of PPR transcripts but not of WHY3. Although WHY3 transcript levels were not significantly affected by miR840 overexpression, its protein synthesis was strongly reduced. Mutating the cleavage sites or replacing the target sequences abolishes the miR840-mediated degradation of PPR transcripts and inhibition of WHY3 translation. In support for this, concurrent knock-down of both PPR and WHY3 in the WT resulted in the senescent phenotype resembling that of the miR840-overexpressing mutant. This indicates that both PRR and WHY3 are targets in the miR840-regulated senescent pathway. Moreover, single knockout mutant of PPR or WHY3 shows a convergent up-regulated subset of senescence-associated genes, which are also found among those induced by miR840 overexpression. Our data provide evidences for a regulatory role of miR840 in plant senescence.

Keywords: senescence; genetics; post-transcriptional control; Arabidopsis; microRNA
Introduction

Senescence is the last developmental stage of whole plants or their organs, and is often associated with a transition, which can be also stimulated by environmental stress. In monocarpic crops, premature senescence leads to the reduction of product yield and postharvest quality. During plant senescence, genes coding for proteins related to autophagy, chlorophyll and lipid catabolism, carbohydrate and nitrogen transport, as well as those involved in generation of reactive oxygen species are up-regulated, whereas others related to protein synthesis and maintenance of mitochondrial or chloroplast functions such as light harvesting, carbon fixation and photorespiration are down-regulated (Lim et al., 2003; Guo and Gan, 2014). Global transcriptome analyses in Arabidopsis showed that approximate 12-16% genes are regulated differentially in senescence-related physiological and pathological processes, indicating the occurrence of extensive transcriptional reprogramming during plant senescence (Guo et al., 2004; Zentgraf et al., 2004; Buchanan-Wollaston et al., 2005; Breeze et al., 2011). This involves tight control by a number of transcription factors (TFs), epigenetic modifications and small non-coding RNAs (Schippers, 2015; Kim et al., 2016; Ren and Miao, 2018; Woo et al., 2019b).

MicroRNAs (miRNAs) are a class of highly conserved endogenous small non-coding RNAs (usually 20-24 nt). Since identification of the first miRNA, lin-4, in Caenorhabditis elegans (Lee et al., 1993), thousands of miRNAs have been identified in animals and plants, and showed manifold roles in controlling diverse biological processes (Ameres and Zamore, 2013; Dexheimer and Cochella, 2020). In plants, multiple factors contribute to the biogenesis, conversion, mobilization and action mechanisms of miRNAs, and in turn, miRNAs control cognate target genes through transcript cleavage and translational repression (Rogers and Chen, 2013; Xie et al., 2015; Yu et al., 2017). Fewer miRNAs with particular link to the regulation of plant senescence have been functionally characterized (Woo et al., 2019a). One example is miR164 which targets the NAC domain-containing proteins such as ORE1 and NAC1 to regulated leaf senescence and cell death during development (Kim et al., 2009; Li et al., 2013). Another microRNA, miR319,
negatively controls a set of TCP (TEOSINTE BRANCHED/CYCLOIDEA/PCF) transcription factor genes, which regulate biosynthesis of the hormone jasmonic acid, to affect leaf development and senescence progression (Schommer et al., 2008). Recently, by using high-throughput smallRNA sequencing strategies, a number of senescence inducible miRNAs in rice, maize and Arabidopsis plants are also discovered (Xu et al., 2014; Thatcher et al., 2015; Qin et al., 2016; Wu et al., 2016). These researches provide large data sets of miRNAs associated with developmental and senescent stages, and in response to nutrition availability or stress conditions. However, specific role of miRNA in controlling senescence of an organ is rarely reported.

Among the senescence-associated miRNAs in leaves (Xu et al., 2014), miR840, is firstly identified in a previous high-throughput pyrosequencing (Rajagopalan et al., 2006), which appears only in genomes in cruciferous plants of the genus Arabidopsis thus considering as an evolutionary young microRNA. A canonical candidate target gene of miR840 is a WHIRLY3 (WHY3), which is a less-studied member of the three-gene family of single-stranded-DNA-binding proteins in Arabidopsis (Cappadocia et al., 2013), The WHIRLY family includes the well-known leaf senescence regulator WHIRLY1 (WHY1) (Miao et al., 2013), the closest paralog of WHY3. However, the function of miR840 is still unclear.

The locus Ath-miR840 (At2g02741) is located within the 3’UTR region of the protein-coding gene PPR (At2g02750), overlapping with the distal portion of the 3’UTR from the opposite strand-encoded gene WHY3 (At2g02740), both being predicted targets of miR840 (Rajagopalan et al., 2006). This special locus arrangement categorizes miR840 into the G3A group of miRNAs, which qualitative and quantitative analysis by sequencing are often hindered by the overlapping or adjacent gene transcripts (Armenta-Medina et al., 2017). Here, we demonstrate that miR840 regulates the onset of plant senescence via targeting PPR and WHY3 in two different manners by degrading PPR transcripts and inhibiting the WHY3 translation concurrently. Neither PPR and WHY3 have been implicated in plant senescence so far, but our analysis suggests that they might
act in concert to negatively regulate this process since the WHY3 and PPR double mutant (kdwhy3 appr) resembles the early senescence phenotype observed in the miR840 overexpression mutant.

Results

MiR840 is processed by three Dicer-like ribonucleases (DCLs) with various efficiency in Arabidopsis

The Arabidopsis miRNA840 precursor (pre-miR840) gene was predicted to be located within a PPR and WHY3 cross-locus (Figure 1A-B), and belonged to group G3A miRNAs (Rajagopalan et al., 2006; Lepe-Solter et al.]. The abundance of the mature miR840 was reported to be reduced by about 0.9-fold in the dcl1 mutant embryos (Nodine and Bartel, 2010; Armenta-Medina et al., 2017). Accordantly, mature miR840 was hardly detected by RNA gel blot analysis in rosettes of the dcl1 mutant, as compared with the small interfering RNA siRNA1003 serving as a control, which is known not to be affected by dcl1 mutation (Figure 1C, left panel). To test whether the other three Arabidopsis DCL genes are involved in the production of miR840, we quantified mature miR840 levels additionally in the dcl2, dcl3, dcl4, dcl4-2t single and dcl2 dcl4 (dcl2&4) double mutants (Pelissier et al., 2011) using stem-loop semi- and qRT-PCR. Except for the dcl3, in which the miR840 level was comparable to that in WT plants, dcl2 and dcl4 showed a decrease in miR840 abundance approximately 39% and 73% relative to the WT, respectively. The double mutant dcl2/dcl4 exhibited a reduction of miR840 levels by 83% as compared to the WT (Figure 1C-D). As controls, the expression of the known DCL1-dependent miR173 was unaffected in the dcl2, dcl3, dcl4 single and dcl2 dcl4 double mutants, whereas the expression of the DCL4-dependent miR839 was strongly declined in dcl4 (by 95%) and in dcl2 dcl4 (by 97%), as well as in the dcl2 single mutant (by 61%) (Figure 1C-D) in consistence with a previous study (Pelissier et al., 2011). Therefore, we conclude that the production of the mature miR840 is dependent mainly on DCL1 and to a lesser extend also on DCL2 and DCL4.
MIR840 expression in rosettes reaches its maximum at the onset of plant senescence

To identify the role of miR840 in regulating plant senescence, we first analyzed the tissue-specific expression of the miR840 precursor (pri-miR840) together with the target genes PPR and WHY3 in young (3-week-old) and aging (13-week-old) plants (Figure 2A & B, respectively). The highest expression levels of these three genes were found in flowers and siliques of 13-week-old plants, in which the abundance of pri-miR840 in the reproductive organs was about ten-fold higher than in the vegetative organs (Figure 2B). The expression levels of pri-miR840 in rosettes of 3-week-old seedlings and 13-week-old plants were comparable, whereas PPR and WHY3 showed a decreased (~ -5-folds) and enhanced (~ +2.5-folds) expression with increasing age, respectively (Figure 2A & B).

To monitor the transcript profiles of miR840 and PPR and WHY3 during plant development and aging, we weekly sampled rosettes from week 5 to week 13. During this period, week 10 marked the senescent initiation stage under our experimental conditions, with activation of a senescence-associated marker gene SAG12 (Figure 2C). Both PPR and WHY3 displayed an antagonistic expression pattern from week 9 on. Interestingly, this time point marked the highest expression of PPR throughout all developmental stages, preceding a similar expression profile of miR840 being one week delayed and coincident with the senescent initiation (Figure 2C). The transcript profiles of mature miR840 as well as of PPR and WHY3 in 9- and 10-week-old rosettes were additionally confirmed by Northern blot hybridization (Figure 2D). Furthermore, we also determined the levels of mature miR840 as well as PPR, WHY3 and SAG12 transcripts during the aging of rosette leaves (with leaves from different positions) and in 4-sectioned leaf segments from yellowish tip to green base of the single 7th leaf of 11-week-old plants (Figure S1 and S2, respectively). Similarly, a significant elevation in miR840 abundance was associated with the onset of leaf senescence as indicated by an up-regulation of SAG12 (Figure S1C). While PPR expression could be negatively correlated with miR840 expression during plant aging, this was
not true for WHY3 (Figure S1B and S2C). These data suggest a possible involvement of miR840 in plant senescence regulation.

**Loss-of-function and gain-of-function analysis indicates a crucial role of miR840 in plant senescence regulation**

Two homozygote T-DNA insertion lines, SALK_038777 and SAIL_232_F08 were employed for further analysis. Both lines can be considered as MIR840 mutants inserted at promoter position (Figure 3A and Figure S3), but also disrupt the PPR ORF. The T-DNA insertion at position -767 bp (SALK_038777) reduced the miR840 level about 95 folds as compared with the WT, whereas the insertion at -384 bp (SAIL_232_F08) drastically enhanced miR840 expression up to approximately 45-fold as revealed by northern blot and qRT-PCR analysis from rosettes harvested at the onset of plant senescent stage (week 10). Thus, we considered SALK_038777 as a miR840-knockdown and SAIL_232_F08 as an overexpression line for this study (Figure 3A). However, it is not yet clear how miR840 expression is affected by the T-DNA-insertions (Figure S3).

Phenotypically, both mutants displayed contrasting leaf development and senescence onset. The SALK_038777 with lower miR840 expression showed a stay-green phenotype even superior to the WT, whilst the SAIL_232_F08 with miR840 overexpression exhibited a strong early senescence-like phenotype (Figure 3B). Qualitative and quantitative determination of senescence related parameters, such as leaf yellowing (Figure 3B), photochemical efficiency of photosystem II F/Fm and leaf ion leakage (Figure 3C) suggested that miR840 has a strong impact on plant development at the later stages (at about 10 weeks). The observed phenotypes were stable and could be confirmed by further measurement with up to 7th generation of the mutant plants (Table S1).

To verify the function of miR840 in plant development and senescence observed from the T-DNA insertion lines, we further generated transformants in Arabidopsis plants ectopically
expressing either the pri-miR840 and a tandem antisense (target) mimicry of miR840 (9x miR840am) (Figure 4A; Figure S4A and S4B). By stem-loop RT-qPCR we found the pri-miR840 OE lines accumulating 289- to 1100-fold more miR840 transcripts when compared with WT, while in the antisense mimicry lines mature miR840 decreased 3-10 fold of WT levels (Figure 4B). Phenotypic analysis revealed that the overexpression of miR840 was indeed associated with early senescence, whilst the knockdown of miR840 by antisense mimicry delayed plant senescence, as indicated by measurements of leaf yellowing, Fv/Fm index, total chlorophylls and total carotenoids contents (Figure 4C and 4D).

The phenotypical differences observed in the T-DNA KD and OE mutants of miR840 were correlated with senescence-associated gene expression as demonstrated by RT-qPCR of 50 senescence-related genes (Figure S5) known to be directly involved in plant senescence or cell death and DNA damage/repair processes. The results showed that the expressions of these genes were differentially affected in the miR840 mutants as compared to WT, albeit to different degrees (Figure S5B). In the miR840-overexpression mutant SAIL_232_F08, a strong increase in gene expression was observed for WRKY53, WRKY33, SIRK, SAG101, SAG12, and PDFs. Consistently, a significant decrease of the gene expression was detected for WRKY53, WRKY33, SAG101 and PDFs genes in the miR840-knockdown mutant. Interestingly, the expression of PR1 gene was increased in SALK_038777 but decreased in SAIL_232_F08 plants, hinting at a possible crosstalk between senescence and the plant defence response. Moreover, the expressions of SPO11-2 and RAD52, two genes functioning in double-stranded break (DSB) related DNA damage and repair mechanism, were up-regulated in SAIL_232_F08 OE but down-regulated in SALK_038777 KD mutants (Figure S5B). Taken together, we conclude that miR840 represents a master-regulatory microRNA in reprogramming cellular pathways to enhance or initiate plant senescence.

MiR840 induces atypical cleavage of its target mRNAs
miR840 is transcribed in the same orientation as PPR and both 3’-UTRs of PPR and WHY3 are predicted targets of miR840* and miR840, respectively (Figure 1A). To detect the miR840-target sites in PPR and WHY3 transcripts, we performed a 5’-RLM-RACE experiment using total RNA extracted from rosettes of WT plants at the senescence onset stage when miR840 was highly accumulated. The efficiency of reverse transcription reaction of the adaptor-ligated cDNA was exemplified by amplification of a reference gene AtUBQ13 and an extremely lowly expressed gene AtSUC7 (Figure S6A), whereas miR164-guided cleavage of NAC1 transcript (Guo et al., 2005) served as a positive control (Figure S6B). Surprisingly, sequencing of the cleaving products of both PPR and WHY3 transcripts revealed their target sites outside the conventional admitted region of the miR840 complementary sequences. In WHY3 it was found to be 9 bases downstream (close to the polyA-tail) of the miR840 binding sequence, whereas in PPR transcript it was located 22 bases downstream of the miR840* pairing region (Figure S6B and S7). Such noncanonical cleavage events have also been reported in other G3A type microRNAs, such as miR844-targeted CDS3 (Lee et al., 2015).

The target sites were further verified using an in vitro mutagenesis experiment. A reporter plasmid was constructed by cloning synthetic oligonucleotides, resembling the 78-nt-region carrying the miR840-targeting sequence or bearing a mutated targeting site, into a CaMV35S-promoter-driven GUS-expression vector (Figure 5A). A 35S promoter-driven pri-miR840 expression plasmid was used as an effector and transferred, in combination with the respective reporter plasmid, into Nicotiana benthamiana by Agrobacterium-mediated infiltration (Figure 5B). Single infiltration of the reporter plasmid showed slightly decreased GUS transcription when it was fused with the original WHY3 or PPR 3’-UTR as compared to that without fusion R (0). But both fusion constructs did grant strong GUS staining (Figure 5C). After mutation of the target sites of miR840, the GUS transcripts were further reduced in both fusion cases and their GUS staining signals became moderate as compared with R (0) (Figure 5C). Nevertheless, co-infiltration with the effector construct caused a drastic reduction in GUS
transcript levels as well as the GUS staining signals to ~ 20% of the respective single construct with the original WHY3 or PPR 3’-UTR. However, such reduction effects caused by co-infiltration with miR840 did not occur if the PPR or WHY3 target site were mutated, which confirms that the determined target sites are no artefacts (Figure 5C). Following these data, we conclude that miR840/miR840* targets the overlapping 3’UTR region of WHY3 and PPR transcripts, respectively, but with atypical target sites outside the respective pairing region (Figure S7).

**MiR840 targets PPR post-transcriptionally but WHY3 is inhibited translationally**

In the next step, we further checked how miR840/miR840* affects PPR and WHY3 expression in planta. Unexpectedly, the WHY3 transcript level was not affected in the two T-DNA lines, SALK_038777 and SAIL_232_F08 as determined by RT-qPCR analysis (Figure 6A). In contrast, the PPR expression was conversely regulated as expected in both the two T-DNA mutant lines (Figure 6A) as well as in the miR840 mutants pri-miR840 OE and 9x miR840am (Figure 6B). These results indicated a strong negative correlation between the PPR expression and the miR840 levels.

To ascertain that PPR is indeed post-transcriptionally regulated by miR840*, we overexpressed PPR with or without the 3’UTR region in the two T-DNA lines, as well as in WT. Again, the WHY3 expression level was not affected in all transgenic plants (Figure 6C) and a strong reduction of PPR transcript levels was found in transformants harbouring the 3’UTR-containing construct, while the reduction of PPR transcripts was protected in transformants harbouring the 3’UTR-less construct (Figure 6C). Moreover, in transgenic plants expressing the 3’UTR-containing construct, the PPR expression levels varied depending on the genetic backgrounds, generally negatively correlating with miR840 expression (Figure 6C).

Even though PPR and WHY3 are both targets of miR840, the WHY3 transcript level was not affected in the two T-DNA lines, SALK_038777 and SAIL_232_F08 (Fig. 6A). We assumed that
the miR840 might then interfere with the WHY3 translation. To test this hypothesis, we performed western blot analysis using an antibody against a specific WHY3 peptide (Fig. 7A). As a result, the protein level of WHY3 in the miR840-OE (SAIL_232_F08) was much lower than that in both the WT and the miR840-KD (SALK_038777) (Figure 7A). To verify the miR840 pairing effect, we deployed three constructs in either the WT or the two T-DNA mutant backgrounds to express estradiol inducible WHY3-luciferase fusions with the WHY 3’-UTR sequences (UTR) or its cleavage-site-mutated form (UTRm) in the pMDC7 vector (Figure 7B). The WHY3-luciferase without WHY 3’-UTR (CK) served as a control. Induced expression of the recombinant fusion proteins was monitored during 1 – 48 h post induction (hpi) in the WT plants transformed using the UTR construct (Figure 7C, left panel). Without the induction, the fusion protein expression levels were very low in the UTR-harboring plants regardless of the genetic backgrounds, whilst at 24 hpi the WT plants and the miR840-KD showed strong signals in the western blot (Figure 7C, right panel), but not in miR840-OE mutant lines, suggesting an translationally inhibitory effect of miR840.

WHY3 transcript levels in WT plants transformed with the three inducible constructs were induced up to 2 - 6 folds by estradiol treatment, and comparable among the two constructs, UTR and UTRm (Figure 7D). However, a significant increase in miR840 levels after estradiol treatment was also detected in both UTR- and UTRm-expressing plants, but not in the CK plants (Figure 7D). In both cases, it was probably caused by a homolog seed sequence of 3’-end miR840 located at the WHY3 3’-UTR (4 bp difference out of 22 bases to miR840, Figure 1B) that could be mis- amplified by the stem-loop qRT-PCR. Therefore, it could be considered as a background noise signal. Nevertheless, the activity of firefly luciferase was gradually increased in all the three types of UTR plants after estradiol induction, but much more pronounced in plants harboring Tnos and UTRm constructs than in those with UTR (Figure 7D). Thus, under WT background, expressed fusion with the original 3’-UTR of WHY3 resulted in inhibition of protein synthesis, whilst this inhibitory effect was dismissed, when the miR840-targeting site at the
3’-UTR was mutated.

We further compared the 3’UTR effect on ectopic expression of firefly luciferase (LUC) activities in the two T-DNA insertion lines with endogenous accumulated or depleted miR840 level. Again, detection of miR840 via stem-loop qRT-PCR posed a background signal in both lines, quantitatively similar to that in the UTR- or UTRm-expressing WT shown in Figure 7D.

Yet, the SAIL_232_F08 plants did have noticeably higher miR840 accumulation than the other line (Figure 7E, left panel). As expected, in the miR840-OE SAIL_232_F08 LUC activities were lowly detectable regardless of induced conditions, indicating that the endogenous miR840 could have inhibited the induced expression of the WHY3-LUC fusion (Figure 7E, right panel). On the contrary, in SALK_038777 after estradiol treatment, LUC activity was markedly increased (Figure 7E). Theses results supported for an inhibitory effect of the fusion reporter by miR840.

Taken together, we demonstrate that both WHY3 and PPR are targeted by miR840/miR840* at their 3’-UTRs, respectively. Whereas pairing of miR840* to the PPR mRNA leads to its degradation, miR840 inhibits the protein synthesis of WHY3.

Both PPR and WHY3 are regulators of senescence with common downstream genes

To investigate the mode of action of miR840-WHY3 and miR840-PPR in plant senescence, a T-DNA insertion line of WHY3 (Salk_005345C) was obtained, in which the T-DNA insertion at -140 bp upstream of its start code (ATG) (Figure S4C) resulted in a 20-fold decrease in WHY3 expression (Figure 8A, left panel insert; Figure S8B and C). This mutant line was assigned kdwhy3. In parallel, transgenic plants using a PPR antisense construct (designated as appr) was generated, in which PPR expression was strongly depressed as compared to WT (Figure S8A and C). However, neither kdwhy3 or appr plants showed accelerated senescent phenotypes as compared to WT (Figure S8D to G), suggesting they worked in concert to initiate the onset of senescence. Therefore, we further generated double mutant by crossing of kdwhy3 x appr. The homozygote double mutant plants kdwhy3 appr selected from a F3 generation were employed for
further analysis. As assumed, the double mutant showed now an early senescence phenotype similar as that observed in the miR840-overexpression lines (Figure 8A and B; Figure 3B and C).

Moreover, to rescue the early senescence phenotype in SAIL_232_F08 OE mutant, we stably overexpressed PPR alone (oePPR) or together with WHY3 (oePPR oeWHY3) in this background (Figure 8C and D). Only transgenic plants overexpressing both PPR and WHY3 genes could rescue the early senescence of the mutant comparable to the WT plants, whilst overexpression of the PPR alone had only a very weak rescue effect (Figure 8C and D).

The double mutant kdwhy 3appr does not only resemble the early senescence phenotype of the miR840-overexpression plants, but also displayed a similar expression profile of downstream genes known to be involved in senescence, cell death and DNA damage repair (Figure 9A to C, and Figure S5). Several of these genes also showed similar expression patterns in the single kdwhy3 or appr plants (Figure S9D and E). Notably, similar regulation of CAMTA3, AHK3 and genes coding for a pyruvate decarboxylase (AT5G01320) and a glutamine synthase (GLN1) were found among kdwhy3, appr and kdwhy3 appr transgenic plants as well as in the miR840-overexpression lines (Figure S9D, E and Figure S5). Besides that, arginine-tRNA protein transferase gene (DLS1), RLP27, ANAC053, COR15B, NEET, AT4G22620, WRKY70 and APG7 genes shared the similar expression patterns in appr and the the miR840-overexpression lines (Figure S9E; Figure S5), and copper amine oxidase (AT4G12290), COR78, ARF2, UBA2A, ANAC092, ARF1, ANAC029, COR15A, WRKY70, MYB34, LEA hydroxyproline-rich glycoprotein (AT1G17620), B12DP and Copia-like retrotransposon (AT5G35935) genes exhibited similar expression patterns in kdwhy3 and the miR840 overexpression lines (Figure S9E; Figure S5).

We conclude that both PPR and WHY3 are targets of miR840-mediated senescence pathway, and simultaneous repression of both genes by miR840 reprograms the expression of a subset senescence-related genes, consequently leading to the onset of plant senescence (Figure 9).

Discussion
miR840 represents an evolutionary young microRNA with a special targeting configuration

The special genomic arrangement of the miR840 locus at the convergent region between 3’UTRs of PPR and WHY3 makes it possible that miR840 may be expressed from its own promoter or generated from 3’UTR of a PPR transcript. Our results indicate that miR840 is mainly produced by Dicer-dependent pathways. Among the four members of the Dicer family in Arabidopsis, DCL1, 4 and 2 are contributing to the processing of the mature miR840 (Figure 1). Although DCL1 can be considered as the major dicing enzyme for miRNA biogenesis (Reinhart et al., 2002), DCL4 is also involved on generation of evolutionary young miRNAs (Rajagopalan et al., 2006). Both DCL4 and DCL2 preferentially produce 21- and 22-nt siRNA from endogenous or viral or transgene, respectively (Borges and Martienssen, 2015; Taochy et al., 2017). While the effect may not be significant, it is interesting here that miR840 (22-nt) production is also partially dependent on DCL2, but a detailed analysis on the RNA species produced by different DCLs is necessary in helping to unveil the underlying mechanism.

Both strands of the duplex miR840 can bind to the mRNAs of WHY3 or PPR, yet the former pairings yield perfect matches but the latter ones consist of 4 mismatches (Figure S7). Such configuration also means that targeting to WHY3 and PPR by mature miR840 may involve an unconventional mechanism, because perfectly matching between miRNA and target sequence is usually associated with cleavage. However, we observed translational repression as the predominant mechanism to prevent WHY3 expression, and cleavage for the PPR transcript despite of the 4 mismatches. Our data revealed furthermore that miR840-guided target sites in both WHY3 and PPR transcripts located outside of the predicted pairing regions (Figure S6), with two distinct consequences - translational inhibition for WHY3 and mRNA degradation for PPR. Similar cleavage events are also reported for miR844 which induces cleavage of its target CDC3 transcript at 6, 12, 21, 52 nt upstream of the predicted target sites, resulting in the instability of the mRNA (Lee et al., 2015). It is also noteworthy that the target site in WHY3 transcript is close to its PolyA tail (33 nt upstream, Figure S7B). Whether that accounts for the inhibition of
translation efficiency is currently not clear. However, the widespread alternative lengthening of 3’ UTRs in most protein-coding genes and long non-coding RNAs are known to affect the functional stability, localization and translation efficiency of the RNAs. (Elkon et al., 2013; Chen et al., 2018). In fact, WHY3 transcript exists in two 3’UTR by alternative polyadenylation (APA): the short one is 242 nt without the miR840 targeting region, while the long one is 563 nt in length (Figure S7B). Further clarification of the regulatory role of WHY3 APA may be needed.

Target prediction using psRNATarget website with low stringency resulted in 121 and 128 candidate transcripts for the 22 nt miR840 and the 21 nt miR840*, respectively (Supplementary data set1). However, these target genes were not verified in the present study, and not reported in other publications concerning miR840 (Rajagopalan et al., 2006; Nodine and Bartel, 2010).

So far, miR840 is found only in Arabidopsis and close relatives, thus it appears to be an evolutionary young microRNA (Rajagopalan et al., 2006). The origin of miR840 may be of general interest in future studies (Cui et al., 2017), owing also to its special target configuration.

The miR840-PPR/WHY3 module functions in plant senescence

During normal growth in Arabidopsis, accumulation of miR840 is found to associated with the development of senescence symptoms (Figure 2). Whereas knocking down miR840 could delay plant senescence, overexpression of miR840 enhances the senescent phenotype (Figure 3; Figure 4). At the molecular level, miR840 targets a convergent gene pair PPR and WHY3 for either transcript degradation or translational repression, respectively and thereby reprograms many senescence-associated downstream genes (SAGs) (Figure S5; Figure S6). These include developmental signal-related SAGs like WRKY53 (Miao and Zentgraf, 2007), SAG12 (He and Gan, 2002), SIRK (Robatzek and Somssich, 2002), SPO11-1, 2, 3 (Hartung et al., 2007) and RAD52 (Samach et al., 2011), as well as environmental stress-induced SAGs such as pyruvate decarboxylase (Kursteiner et al., 2003), glutaminase synthetase (Li et al., 2006), CAMTA3 (Nie et al., 2012), AHK3 (Kim et al., 2006), RLP27 (Wang et al., 2008), COR78 (Yang et al., 2011b), WRKY70 (Besseau et al., 2012), SAG101 (Feys et al., 2005), WRKY33 (Birkenbihl et al., 2012),
EIN2 (Kim et al., 2009), PDFs (Liu et al., 2007) and PRI (Uknes et al., 1992; Epplle et al., 1997; Thomma et al., 2002). Some of these downstream genes are involved in the response to wounding, jasmonic acid, fungus, water deprivation, cold and acid stresses (Alonso et al., 1999; Kim et al., 2006; Kim et al., 2009; Yang et al., 2011a; Thomas, 2013), implying that the miR840-induced early senescence may affect biotic and abiotic stress responses.

miR840-mediated senescence onset requires joint repression of both WHY3 and PPR, due to the fact that single mutation of either WHY3 or PPR is not sufficient to mimic the miR840 overexpression phenotype. However, the double mutant kdwhy3 appr displays a similar phenotype as the miR840 overexpressing plants, and that only co-expression of both in this mutant background can rescue the early senescent phenotype (Figure 8A-B). Consistently, several SAGs were found to share similar regulated expression pattern among kdwhy3, appr, kdwhy3 appr and miR840 overexpressor mutants (Figure S5 and Figure S9).

Interestingly, both PPR and WHY3 were not known for their involvement in plant senescence in previous studies, possibly owing to the weak phenotype of respective single mutants. PPR belongs to the pentatricopeptide repeat superfamily which encodes ~ 450 proteins in Arabidopsis. PPRs are RNA-binding proteins and are found in complexes of organelle mRNA-editing machineries in plants, which play roles in the regulation of photosynthesis, respiration, as well as in plant development and environmental responses (Barkan and Small, 2014). On the other hand, WHY3 is a member of the three-gene family of WHIRLY single-stranded DNA binding proteins in Arabidopsis (Cappadocia et al., 2013) and is believed to localize dually in nucleus or plastid and mitochondria (Marechal et al., 2009; Jiang et al., 2017; Golin et al., 2020). Its closest homolog WHY1 represses the senescence marker gene WRKY53 and coordinates leaf senescence in a developmental stage-dependent manner (Miao et al., 2013; Huang et al., 2017; Ren et al., 2017; Huang et al., 2018; Lin et al., 2019).

Taken together, this work adds new regulatory aspects in plant development and onset of senescence depending on the evolutionary young miR840. Its ability to accelerate plant
senescence upon its accumulation depends mainly on joint repression of the neighboring convergent genes PPR and WHY3 by targeting their overlapping 3’ UTRs (Figure 9).

Materials and Methods

Plant materials and growth conditions

Plants of Arabidopsis thaliana (ecotype Columbia) were grown in a growth chamber under long (16 h light/8 h dark) or short (8 h light/16 h dark) illumination condition as described before (Miao et al., 2013). Phenotyping was assessed under the same illumination condition in all cases as indicated appropriately in the Results section.

T-DNA insertion lines SAIL_232_F08 and SALK_038777 for MIR840 (AT2G02741), Salk_005345C for WHY3 (AT2G02740) were ordered from NASC (http://arabidopsis.info/BasicForm) and confirmed by genotyping PCR with the primers suggested by the T-DNA Express Tool and by qRT-PCR with gene specific primers (Table S2). The homozygote dcl1, dcl2, dcl3, dcl4, dcl4-2t and dcl2dcl4 mutants were kindly provided by other scientists. Plasmids for Overexpression-PPR (35S::PPR) and overexpression-PPR-3’UTR (35S::PPR-UTR) were created by inserting the PPR coding sequence alone or with its 3’UTR into the destination vector pB2GW7 by GATEWAY cloning technology. Transgenic lines appr/WT and kdw3 appr were created by transforming WT and kdw3 plants with the antisense-PPR (appr) plasmid constructed on pB2GW7 by Gateway-cloning the complementary PPR CDS (Figure 8A). Transgenics were selected by spraying seedlings with 0.1% (w/v) glufosinate ammonium and confirmed by semi-qPCR and qRT-PCR, and the T3 homozygous plants were used for phenotype observations. The pri-miR840 OE (Figure 4A) transgenic plants were generated by transformation of WT plants using pCBIM (Ren et al., 2012) harboring the 226 bp pri-miR840 sequence (Figure 1A).

The tandem mimicry miR840 inhibitory vector was constructed in such a way that a nine-tandem-repeated miR840 22-nt sequence together with insertion of three extra nucleotides in
the 10\textsuperscript{th} and 11\textsuperscript{th} position to form an unpaired inhibitory loop after transcription was first assembled (Figure 4A; Figure 4SA) and inserted reversely into the MCS site of the pCBIM vector, as described in detail previously (Jiang et al., 2014). The resulting plasmid and the pCBIM empty vector were used to generate the 9x miR840am and the control transgenic plants on the WT background, respectively. The T1 seedlings were screened on plates containing 50 μg/ml hygromycin and the positives were checked with RNA gel blot (Figure 4B). Transgenic plants of 9x miR840am showing an expected interfering RNA species (marked with a red arrow in Figure 4B) were selected for further experiments (Figure 5h).

RNA isolation, northern blot, real-time quantitative PCR (qRT-PCR) and stem-loop qPCR

Total RNA was extracted using Trizol reagent (Invitrogen). Twenty micrograms of total RNA were used for long or small RNA isolation as described before with minor modification (Miao et al., 2004). Briefly, high molecular weight RNA was selectively precipitated from the total RNA by addition of one volume of 20% PEG with 1M NaCl (Llave et al., 2002). The supernatants enriched with low molecular weight RNA (80-100 μg) was then separated on 17% denaturing polyacrylamide gels and electro-blotted to a Hybond-N\textsuperscript{+} membrane for small RNA detection. Probes for miR840, siRNA1003 and U6 were synthetic oligonucleotides complementary to their sequences and end-labeled with \textsuperscript{32}P-ATP using T4 kinase (Fermentas). Probes for full-length PPR or WHY3 CDS were \textsuperscript{32}P random-primer-labeled complementary DNA. Unincorporated nucleotides were removed using G-25 spin columns (Amersham) according to the manufacturer’s instruction. Blotting conditions were previously described (Miao et al., 2004). Membranes were exposed to X-ray films and the ethidium bromide stained rRNA and tRNA was used as loading amount control.

For qRT-PCR, first-strand cDNA was synthesized and detected using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) and SYBR Green PCR Master Mix (Invitrogen). Quantification of expression were based on reference genes ACTIN 2 or PP2A when
developmental stages were compared. Gene-specific primers for MIR840, PPR and WHY3, as well as the fifty selected downstream genes were list in Figure S5A, and their specificity and amplification efficiency were confirmed by examining the melting curves.

For stem-loop qPCR analysis of microRNA, reverse transcription was done with miRNA first strand cDNA synthesis kit (Vazyme biotech, MR101-02) according to the manufacturer’s instruction. The specific stem-loop primer was designed by the online miRNA primer design software provided by the manufacturer, such that the RT primer for miR840 and U6-29 contained with their 5’ 6 nt annealing to the respective mature microRNA 3’ 6 nt, and the qPCR primers consisted with a universal reverse primer together with the microRNA-specific primers (Kramer, 2011). The primer sequences are given in Table S3.

5’RLM-RACE and sequencing

Using T4 RNA ligase 1 (NEB, M0437M), total RNA was isolated from 10-week-old or senescent rosettes of WT plants were ligated to a specific oligo adaptor at their 5’terminal, subsequently converted to first-strand cDNAs with a RevertAid first strand cDNA synthesis kit (Thermo Scientific™, K1622) following the manufacturer’s instruction. The detail procedure was described in supplementary method and Figure S6A. The RNA oligo and primers used in 5’RLM-RACE are listed in Table S4.

GUS reporter assay for miR840-guided gene repression

A cloning plasmid pGWB433-MCS-GUS was constructed (Supplementary Method). This empty vector was assigned as reporter plasmid zero R(0) in Figure 5A. Oligonucleotides with an added ATG reminiscent of the 78 bp in the WHY3 3’UTR region overlapping with PPR, containing either the original (CACT) or mutated (GTGT) target sites of WHY3, were synthesized and inserted into pGWB433-MCS-GUS in frame with the GUS gene to create another 2 reporter plasmids R(W3UTR) and R(W3UTRm), respectively. Similarly, the reverse complementary
sequence of the same 78 bp region was used to generated PPR 3UTR-GUS reporter plasmids R(P3UTR) and R(P3UTRm) containing the original (TGCG) and mutated (ACTA) target sites, respectively (Figure 5A and Figure S7). For construction of the effector plasmid, full-length pri-miR840 sequence was cloned into the MCS of the vector pCBIM (Ren et al., 2012). All constructs were verified by sequencing.

Agrobacterium tumefaciens strain GV3101 harboring individual plasmid was grown overnight in liquid LB medium containing 20 μg/mL Acetosyringone (AS) and a diluted culture with 100 μg/mL AS reaching an OD$_{600}$ 2.0 was harvested for leaf infiltration of one-month-old N. benthamiana plants after washing and resuspension with MES-KOH buffer (pH 5.7) at a concentration of OD$_{600}$ 0.4. For co-infiltration, equal amount of Agrobacterium suspensions was used. Before infiltration of the 3rd leaves of N. benthamiana, the Agrobacterium suspensions was kept on bench for 3 hrs. At 3 days post infiltration, leaf discs were sampled for GUS staining and qRT-PCR verification. The plasmid-based HptII was used as reference gene for expression quantification. The primers used in this experiment are listed in Table S4.

**Inducible luciferase-fusion assay**

To construct the WHY3-LUC fusion, a pENTR/TOPO-WHY3-LUC vector with the 560 bp WHY3 3’UTR sequence (UTR), the mutated version of the 3’UTR (UTRm) or without the 560 bp WHY3 3’UTR sequence were created. The mutated version of the 3’UTR (UTRm) was produced by incorporating substitute nucleotides in the reverse PCR primer. The LUC-UTR or LUC-UTRm cassette, was then isolated by digesting with NotI and EcoRI and sub-cloned into a previously constructed gateway entry vector harboring the full-length CDS of WHY3, to yield pENTR/TOPO-WHY3-LUC-UTR or pENTR/TOPO-WHY3-LUC-UTRm such that the LUC CDS was downstream in frame with the WHY3 CDS linked by 21 bp sequence including the NotI site (Figure 7B). Finally, three binary vectors were generated by LR reaction using pMCD7
(Curtis and Grossniklaus, 2003) and the above entry vectors. All constructs were subjected to sequencing verification.

Transgenic seedlings were selected by spraying with 0.1% (w/v) glufosinate ammonium and identified by semi- and quantitative real-time PCR. Luciferase activity was determined according to the instruction manual of the reporter assay system (Promega, USA) with modifications. Briefly, 100 mg leaf discs were harvested and frozen in liquid nitrogen. After grinding, 100 µL 1x passive lysis buffer was added and mixed vigorously. The samples were incubated for 1 h on ice followed by centrifugation for 20 min at 13,000 x g. The resulting supernatant was diluted 1:5, 1:10, 1:20, 1:40 and put on a 96-well plate. After subsequently adding LARIIL, luminescence was measured in a Flexstation 3 Microplate Reader (Molecular Devices, USA). Total protein in the supernatants were determined by the Bradford method. Experiment was repeated at least three times.

Protein extraction and immunodetection

For total soluble protein extraction, 200 mg fresh leaf materials were batch frozen in liquid nitrogen, ground into powder, resuspended in 100 µL of extraction buffer (100 mM Tris, pH7.2, 10% sucrose, 5 mM MgCl₂, 5 mM EGTA, protease inhibitor) and centrifuged at 15,000 x g for 10 min. The supernatants were used for western blot analysis. Proteins were separated on 10% acrylamide gel and transferred to nitrocellulose membranes by semi-dry blotting. The membranes were blocked for 1 h at room temperature in TBS buffer containing 5% (w/v) non-fat dry milk powder, then incubated either with anti-WHY3 peptide antibody (provided by Prof. Dr. Karin Krupinska, University of Kiel) or with antibody-free PBS solution for 1 h, respectively. Blots were washed in TBST buffer for 10 min (3 times) before incubation with secondary antibody conjugated with a peroxidase. The blots were washed with TBST for 10 min (3 times) and then detected by chemiluminescence.
Measurements of chlorophyll and carotenoid content, chlorophyll fluorescence and membrane ion leakage

The 7th true leaf of a rosette was used for chlorophyll extraction. Chlorophyll a, b, total chlorophyll and total carotenoids contents were calculated according to a previous reported method (Wellburn, 1994). At least 15 plants were determined to calculate the representative mean of the biological sample. In some cases, chlorophyll contents were also detected by using the DUALEX® SCIENTIFIC+ portable plant polyphenol-chlorophyll meter (Force-A, France), and the results were presented in unit μg/cm². Chlorophyll fluorescence was measured from 5- to 13-week-old plants (grown under 8 h light) as described previously (Miao et al., 2013).

The No.7 true leaf in 5- to 13-week-old rosettes was used for measurement of membrane ion leakage as described previously (Miao et al, 2007).

Statistical analysis

Mean values and standard deviations (SD) were calculated in Microsoft Office Excel 2019. Statistical significance among various comparisons were analyzed by one-way ANOVA or pair-wide multiple t-tests using the software Origin 7.5 (OriginLab Corporation, USA). Two asterisks indicate extremely significant differences when \( p \)-value \( \leq 0.01 \), while one asterisk indicates significant differences with a \( p \)-value \( \leq 0.05 \).

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Author contributions
Y.M. and Y.R. designed the project. Y.R., Y.M., W.W., and W.L. performed the experiments, collected data, and analyzed the results. Y.R. and Y.M. wrote the manuscript. D.C. and S.D. contributed to miRNA experimental discussions and manuscript correction.

Competing financial interests
The authors declare no competing financial interests.

Data availability
The authors declare that all data supporting the findings of this study are available within the manuscript and its supporting information is available from the corresponding author upon request.

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Figure 1 Gene locus and DCL-dependency of miR840 in Arabidopsis. (A) The MIR840 is located within a convergent gene pair in chromosome 2. The 226 bp pri-miR840 was shown and mature miR840 sequence was underlined and highlighted. (B) Predicted RNA structure of the PPR 3’UTR, pri-miR840 and WHY3 3’UTR, by RNAstructure© ver.6.0.1. The mature miR840 strand (red) and its pairing strand miR840* (blue) were labeled. (C) Northern blot (leaf panel) and stem-loop semi-qPCR (right panel) detection of miR840 in dcl1, dcl2, dcl3, dcl4, dcl4-2t and double dcl2dcl4 mutant plants. EtBr: ethidium bromide staining. For semi-qPCR, the PCR cycles for amplification are shown beside the gel of each microRNA with unequal numbers indicated.
(D) Stem-loop RT-qPCR showing fold-change of miR840 level in dcl2, dcl3, dcl4 and double dcl2dcl4 mutant plants over the that in WT.

Figure 2 Expression of MIR840, PPR and WHY3 during development and senescent process. (A)-(B) Tissue-specific expression in rosette and root of 3-week-old seedlings and in 13-week-old plants, respectively. (C) Age-dependence expression profile in rosettes. The senescence-associated gene SAG12 was used as a molecular marker of senescent stage of Arabidopsis plants. (D) Northern blot of miR840, PPR and WHY3 transcripts in rosettes of 9- and 10-weeks when senescence is set. EtBr: ethidium bromide staining.
Figure 3 Phenotypical characterization of the two T-DNA mutant lines of Arabidopsis.

(A) Positions of T-DNA insertion and the effect on miR840 expression of the two mir840 mutations. (B) Senescent phenotypes comparing with WT plants. Twelve 10-week-old plants were measured for leaf color scoring under 8 h illumination condition (mean ± SD). Representative photos are shown. (C) Relative photochemical efficiency of photosystem II (Fv/Fm) (left panel) and ratio of the membrane ion leakage (right panel) in rosette leaf No.7 were determined at different developmental and senescent stages. The data are mean ± SD of twelve (for Fv/Fm) or five (for ion leakage) independent measurements. * P < 0.05, ** P < 0.01.
Figure 4 Senescence-related phenotyping of pri-miR840 OE and 9x miR840am transgenic plants. (A) Schema of the expression cassettes for transgenes pri-miR840 OE and 9x miR840am. P35S, CaMV35S promoter; Tnos, NOS terminator. (B) Determination of miR840 accumulation in rosettes of independent transgenic lines, showing fold-change over WT plants. (C) Senescent phenotypes of 6- and 8-week-old WT, pri-miR840 OE and 9x miR840am transgenic plants. Representative 6-week-old plants showing early senescence in the OE transgenics as compared to the others (left panel). Representative 8-week-old plants with ordered rosette leaves were displayed (right panel). (D) Measurement of Fv/Fm value (left panel), total chlorophyll content (middle panel) and total carotenoids content (Cx,c) (right panel) in the 4th rosette leaf of 6-week-old plants. Data represented mean ± SD (n =5).
Figure 5 A GUS report assay demonstrating a site-specific dependency in miR840-guided transcript cleavage. (A) Effector and reporter plasmids designed in the *N. benthamiana* transient assay. The ATG-less GUS cDNA is transcribed downstream of and in frame with a 78 bp short sequence containing the specific miR840 cleavage sites in the 3'UTR region of PPR and WHY3 transcripts, respectively. Nucleotide substitutions are highlighted in red and blue, respectfully. (B) qRT-PCR detection of *pri-miR840* abundance at three days post infiltration, using the plasmid-encoded *NptII* as an internal reference. (C) Transcript level and GUS staining at three days post infiltration. Statistical significance was determined using a paired Student’s t-test with alpha = 0.05; the adjusted p-value is shown above the data (n = 5).
Figure 6 PPR expression is targeted post-transcriptionally by MiR840. (A) Northern blot and qRT-PCR analysis of WHY3 and PPR transcripts in miR840 gain-of-function mutant (SAIL_232_F08) and loss-of-function mutant (SALK_038777) and in WT plants. (B) Fold-change in transcript abundance of PPR in transgenic lines of pri-miR840 OE and 9x miR840am, comparing to the WT. (C) mRNA level of PPR and WHY3 in rosettes of transgenic lines over-expressing PPR CDS either with or without its 490-bp 3’UTR in WT or miR840 mutant background. Data are mean ± SD (n =3). ** p < 0.01.
Figure 7 WHY3 expression is translationally inhibited by MiR840. (A) Western blot showing WHY3 protein level in SAIL_232_F08, SALK_038777 and WT plants. The anti-Rubisco and Coomassie staining were used for loading control. (B) A schema showing the estradiol induced constructs for WHY3-LUC in frame fusions based on the pMDC7 vector. CK: no additional sequence was added after the fusion; UTR: the 560 bp WHY3 3’UTR sequence was inserted downstream of the fusion; m-UTR: the 560 bp WHY3 3’UTR sequence with mutated miR840 cleavage site (nucleotides in red). (C) Time-course of fusion protein accumulation after
estradiol treatment in stably transformed WT plants overexpressing the UTR construct (left panel),
and the 24-h-induced fusion protein levels in the three transgenic plants stably expressing the
UTR construct (right panel). Coomassie staining is used as a loading control. Note that the fusion
protein is repressed in the gain-of-function miR840 mutant (D) Quantification of miR840 and
WHY3 transcript level, as well as luciferase activity in transgenic plants of the WT background
after treatment with estradiol. Note that the luciferase activity is blocked in plants with UTR
construct but not in the UTRm and CK plants. (E) Correlation of miR840 abundance (left panel)
and luciferase activity (right panel) using UTR transgenic plants of the two miR840 mutants. Data
of two independent lines from each are shown. Data are given as mean ± SD of three biological
replicates. * p < 0.05; ** p < 0.01; *** p < 0.001. A.U., arbitrary unit.
Figure 8 Double-knockdown mutants of PPR and WHY3 show similar early-senescence phenotype with the gain-of-function miR840 mutant to a less extent. (A) A kdwhy3 appr double mutant was generated by stable transformation of the kdwhy3 mutant (T-DNA insertion line, SALK_005345C, Figure S4C) with a PPR antisense construct (appr). Representative photos of the rosette leaves are shown (right panel). (B) Determination of relative photochemical efficiency of photosystem II (Fv/Fm) (left panel) and total chlorophyll content in rosette leaf No. 7 (middle panel), as well as of proportion of leaf coloring of the rosette leaves (right panel). The value represents mean ± SD of 12 independent measurements for Fv/Fm and Chl content, and 15 for yellow/green leaf ratio. Significant difference: * p < 0.05, ** p < 0.01. (C) Phenotype analysis
of stable transgenic plants overexpressing PPR (oePPR) or WHY3 (oeWHY3) in the WT background and oePPR alone or together with oeWHY3 in the miR840 gain-of-function mutant SAIL_232_F08. Note that the early senescence phenotype of the SAIL_232_F08 can be restored to that of WT by co-expression of PPR and WHY3. (D) Quantification of relative photochemical efficiency of photosystem II (Fv/Fm) (left panel), and total chlorophyll content (middle panel) in the 6th rosette leaf, as well as SAG12 gene expression in the same plants as in (C). The insert shows transcript levels of PPR and WHY3 in WT plants and the transgenic plants.

Figure 9 A working model showing how miR840 regulates plant senescence in Arabidopsis. Increased expression of MIR840 may be activated by developmental or environmental signals. Processing of pri-miR840 and generation of mature miR840 involve many
factors but are controlled by $DCL1$, $DCL4$ and to a less extent $DCL2$. On the contrary, $DCL3$ is not required for miR840 production. Accumulated miR840 or its star strand is then loaded on ARGONAUTE proteins as part of the RISC (RNA-induced silencing complex) and target to mRNAs of $WHY3$ and $PPR$ by pairing with the complementary region of the respective transcripts. Both miR840 and miR840* are able to bind to $WHY3$ transcripts with perfect match, and to $PPR$ transcripts with four mis-matches, and the binding leads to the cleavage at specific sites downstream of the pairing regions, which are considered as nonconventional. Whereas $PPR$ transcripts are reduced by miR840-guided degradation, $WHY3$ protein synthesis is inhibited by a miR840-mediated mechanism. Target repression by miR840 may include other unproven genes, however, concurrent knockdown of $WHY3$ and $PPR$ is sufficient to mimic the early senescence phenotype of gain-of-function miR840 mutations. Down-regulation of $WHY3$ and $PPR$ provokes up-regulated expression of a set of senescence-associated genes ($SAGs$), which in turn triggers the initiation of senescent progression. The miR840-$PPR/WHY3$ regulatory pathway of plant senescence seems to be a new module limited to plants of the genus Arabidopsis.
Supplementary materials (figure legends, captions of S. Tables and data set)

Figure S1 Expression quantification of miR840, PPR and WHY3 in leaf position of a 13-week-old rosette. The value was displayed as a mean ± SD from three biological replicates.

Figure S2 Differential senescent area in a single leaf and gene expression analysis. * p < 0.05 ** p < 0.01 (n =3).

Figure S3 Distribution of cis-acting elements in the -1000 bp region of MIR840 promoter. The location of each cis-acting element is colored and labeled below the sequences. The T-DNA insertion sites of the gain-of-function (SAIL_232_F08) and loss-of-function (SALK_038777) mutants were indicated by arrow line.

Figure S4 Illustration for mimicry miR840 inhibition, WHY3 knockdown T-DNA mutant and the WHY3 overexpression construct. (A) A working model of the mimicry miR840. The reverse mimicry miR840 finds and anneals to the normal miR840 in vivo, leading to competitive inhibition the functions of the normal miR840. (B) Semi-qPCR to detect the expression level of the 9x tandem mimicry miR840 construct (9x miR840am) in T1 transgenic plants compared to WT control. Red arrow indicates the positive band in different transgenic line. (C) The T-DNA insertion site of the kdwhy3 mutant. (D) The overexpression WHY3 (oeWHY3) construct. P35S, CaMV35S promoter; Tnos, NOS terminator; WHY3, the full length WHY3 CDS.

Figure S5. Quantification of expression of 50 known senescence-associated genes in rosette of the T-DNA insertion lines SAIL_232_F08 (with up-regulated miR840) and SALK_038777 (with down-regulated miR840). Related information of the selected genes with reference is listed in (A). Data represents fold-change over that of WT (n = 3 plants).

Figure S6 Determination of miR840-guided cleavage sites in PPR and WHY3 transcripts using 5’RLM-RACE cloning and sequencing. (A) Qualitative control of RNA preparation, adaptor ligation (left panel) and reverse transcription reaction (RT) in the RLM-RACE system (middle and right). The known high abundantly expressed UBQ13 (middle panel) and lowly expressed gene SUC7 (right panel) were amplified from the cDNA preparations. (B) The WHY3 and PPR RACE products were amplified by 2-round PCR using gene specific primer together with 5’RACE primer (lane 1) and with 5’nested primer (lane 2) subsequently. The NAC1 transcript targeted by miR164 serves as method verification.

Figure S7 Nucleotide sequences of the 3’UTR of PPR (A) and WHY3 (B) showing the localization of the miR840-guided cleavage sites (blue and red arrow) and binding regions of miR840/miR840* (boxed). The 78 nt sequences used for synthetic oligonucleotides in the GUS reporter assay are indicated by brackets below the sequences.

Figure S8 Senescence-related phenotyping of the knockdown why3 (kdwhy3) and antisense-PPR (appr) transgenic lines. (A)-(C) Semi-qPCR and qRT-PCR verification of the transcript level of PPR in the appr transgenic lines and of WHY3 in the kdwhy3 mutant. T3 homozygous plants were used. The value is displayed as a mean ± SD of three biological
replicates. Significant differences were detected by one-way ANOVA test using Origin 7.5 software. ** $P < 0.01$. (D) Comparison of senescent status of appr, kdwhy3 and WT plants grown for 8 weeks in short illumination condition. Rosette leaves were arranged in the order from the oldest to the youngest. (E)-(F) Fv/Fm value and total chlorophyll content of the 7th rosette leaf measured during development. The value is displayed as a mean ± SD of twelve independent measurements. (G) Ratio of categorized leaves in 8-week-old plants. The value represents mean ± SD of 12 independent measurements.

Figure S9 Quantification of gene expression of selected senescence-associated genes in rosettes of kdwhy3appr, kdwhy3 and appr transgenic plants, shown as fold change over that in WT. mean ± SD (n = 3 plants). The list of genes is shown in Figure S5.

Supplementary data set 1. miR840 target prediction by psRNATarget date20200718.

Table S1. Segregation of senescence-like phenotypes in different progeny generations of SALK_038777 and SAIL_232_F08 plants.

Table S2. Primers used for semi- or qRT-PCR analysis of gene expression in this research.

Table S3. Primers used for stem-loop qPCR of miR840 and related vector constructions.

Table S4. Primers used for 5’RLM-RACE reactions and GUS reporter assay.


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