Identification and characterization of new factors in the framework of *Arabidopsis thaliana* miRNA function

Dissertation

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To all the plant-lovers in my family.

Summary

One of the major forces steering differentiation and fate specification is transcription. A main regulator controlling transcript accumulation are small RNAs (sRNAs), acting via silencing of genes through homologous nucleic acids. Within sRNA populations, microRNAs (miRNAs) are amidst the most abundant components, and important factors in regulation of protein coding gene expression in higher eukaryotes. Both their biogenesis and processing are well characterized in animals, and a large number of protein complexes and cofactors involved have been identified and studied. In plants, considerably fewer details are known, and especially the labyrinthine regulation of miRNA biogenesis and function is far from understood.

In recent years, numerous screens were employed to enlighten this labyrinth. Within the frame of this thesis, I characterized three novel cofactors involved in miRNA biogenesis and function that were identified by two such forward genetics screens established in our laboratory, and broadened our knowledge about additional regulatory layers.

The first, luciferase based screen broadly targeted miRNA biogenesis and function and yielded two candidates characterized here, *TRANSCRIPTIONAL DEFECTS OF HPR1 BY OVEREXPRESSION 2* (*THO2*) and the *REGULATOR OF CBF GENE EXPRESSION 3* (*RCF3*). With new and known *tho2* alleles ranging from hypomorphic to null mutants, we found that THO2 is associated with miRNA precursors and their processing, as well as with splicing. This substantiates the connection between miRNA pathways, transcription and splicing, and adds detail to the picture of co-transcriptional miRNA precursor processing. RCF3, on the other hand, proved to influence miRNA levels and action prevalently in young, dividing tissues in the vegetative and reproductive apical region, presumably via affecting the phosphorylation and thus activity of a major miRNA processing factor. These findings not only shift the focus to tissue-biased action as a novel regulatory layer, but also emphasize the importance of chemical modifications of known miRNA co-factors to modulate their function.

The third candidate studied in this thesis, *HAWAIIAN SKIRT* (*HWS*), stems from a screen utilizing miRNA target mimicry to look for modifiers of miR156 function. I genetically and molecularly connect HWS to the general miRNA framework. Function of HWS in this setting appears to be dependent on the activity of its F-box domain, presumably in the context of an Skp-Cullin-F-box (SCF) complex that normally targets substrates for ubiquitination. How exactly HWS functions however remains elusive. Potentially, full characterization of HWS could help explain the principles of target mimicry and uncover a new, additional connection between the ubiquitination machinery and miRNAs.

In summary, the work presented here shows both the potential and the limitations of forward genetic screens to illuminate the rich picture of plant miRNA biogenesis and function. It opens up novel regulatory layers for further, more targeted analysis, and explains the requirements for combined biochemistry, molecular biology and genetics approaches. Finally, broadening the perspective, it suggests that the quest for a detailed picture of the miRNA framework could help us understand the contribution of miRNA regulation and its modulators to adaptation and evolution.

Zusammenfassung

Zelluläre Differenzierung und Zellschicksale werden in großem Maße auf der Ebene der Transkription gesteuert. Die Ansammlung von Transkripten wird zu einem großen Teil durch kleine RNAs (small RNAs, sRNA) reguliert, die über homologe Nukleinsäuren Genprodukte stilllegen können. MikroRNAs (miRNAs) gehören innerhalb von sRNA-Beständen zu den häufigsten Komponenten und sind wichtige Faktoren in der Regulierung der Expression Protein-kodierender Gene in höheren Eukaryoten. Sowohl ihre Biogenese als auch ihre Reifung sind in Tieren gut charakterisiert, und eine große Anzahl daran beteiligter Proteinkomplexe und Kofaktore wurden identifiziert und untersucht. In Pflanzen sind deutlich weniger Details bekannt, und besonders die labyrinthartige Regulierung von MiRNA Biogenese und Funktion sind bei Weitem noch nicht verstanden.

In den letzten Jahren wurden zahlreiche Mutantensichtungen dazu genutzt, Licht in dieses Labyrinth zu bringen. Im Rahmen dieser Dissertation habe ich drei neue Kofaktoren charakterisiert, die an MiRNA Biogenese und Funktion beteiligt sind, und mittels zweier in unserem Labor etablierter Mutantensichtungen identifiziert wurden, was unser Wissen über zusätzliche regulatorische Stufen erweitert hat.

Die erste, auf Luziferase basierte Mutantensichtung fokussierte sich allgemein auf MiRNA Biogenese und Funktion und brachte zwei Kandidaten hervor, die hier charakterisiert werden, *TRANSCRIPTIONAL DEFECTS OF HPR1 BY OVEREXPRESSION 2* (*THO2*) und *REGULATOR OF CBF GENE EXPRESSION 3* (*RCF3*). Mit neuen und bereits bekannten *tho2* Allelen, die sowohl hypomorphe als auch Null-Mutanten umfassen, fanden wir heraus, dass THO2 mit MiRNA Vorläufern und deren Reifung sowie mit Spleißen in Zusammenhang steht. Dies untermauert die Verbindung zwischen MiRNA-Stoffwechselwegen, Transkription und Spleißen, und fügt dem Bild der ko-transkriptionalen MiRNA-Vorläuferverarbeitung neue Details hinzu. RCF3 auf der anderen Seite beeinflusst die Mengen und die Wirkung von MiRNAs, vorwiegend in jungen, sich teilenden Geweben am vegetativen und reproduktiven Apex, mutmaßlich durch Einflussnahme auf die Phosphorylierung und dadurch die Aktivität eines Haupt-MiRNA-verarbeitenden Faktors. Diese Erkenntnisse verschieben nicht nur den Fokus hin zu

Gewebespezifität als neuer regulatorischer Stufe, sondern bekräftigen auch die Wichtigkeit chemischer Modifikationen bereits bekannter MiRNA Kofaktoren zur Modulierung ihrer Funktion.

Der dritte Kandidat, der in dieser Dissertation untersucht wurde, *HAWAIIAN SKIRT* (*HWS*), stammt aus einer Mutantensichtung, der MiRNA Angriffsziel-Mimikry nutzt, um nach Modifikatoren der Funktion von MiR156 zu suchen. Meine Arbeit verbindet HWS genetisch und molekular mit dem allgemeinen MiRNA-Rahmenwerk. Die Funktion von HWS in diesem Rahmen scheint abhängig zu sein von seiner F-box Domäne, voraussichtlich in Zusammenhang mit einem Skp-Cullin-F-box (SCF) Komplex, der Substrate für Ubiquitinierung markiert. Wie genau HWS funktioniert, bleibt jedoch schwer definierbar. Eine komplettierende Charakterisierung des HWS Proteins könnte helfen, die Grundlagen von Ziel-Mimikry zu erklären und eine neue, zusätzliche Verbindung zwischen der Ubiquitinierungsmaschinerie und MiRNAs aufdecken.

Zusammengefasst zeigt die hier vorliegende Arbeit sowohl das Potenzial als auch die Grenzen von Mutantensichtungen als Werkzeug zur Aufklärung der Komplexität der Biogenese und Funktion von MiRNAs. Die Arbeit deckt neue regulatorische Stufen auf für zukünftige, noch gezieltere Analysen, und erklärt die Notwendigkeit von Ansätzen, die Biochemie, Molekularbiologie und Genetik kombinieren. Schließlich, die Perspektive erweiternd, schlägt sie vor, dass das Streben nach einem detaillierten Bild des MiRNA-Rahmenwerks helfen könnte, den Beitrag von MiRNA-Regulierung und ihrer Modulatoren zu Adaptierung und Evolution zu verstehen.

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Publications

Published papers

Francisco-Mangilet AG*****, **Karlsson P***, Kim MH, Eo HJ, Oh SA, Kim JH, Kulcheski FR, Park SK, Manavella PA (2015). THO2, a core member of the THO/TREX complex, is required for microRNA production in Arabidopsis. *Plant J* 10.1111/tpj.12874.

Karlsson P, Christie MD, Seymour DK, Wang H, Wang X, Hagmann J, Kulcheski FR, Manavella PA (2015). KH domain protein RCF3 is a tissue-biased regulator of the plant miRNA biogenesis cofactor HYL1. *PNAS* 10.1073/pnas.1512865112

Manuscript in preparation

Lang P*, Christie MD*****, Dogan E, Hagmann J, Weigel D. - A role for the F-box protein HAWAIIAN SKIRT in plant miRNA function.

Additional manuscripts in preparation not included in this dissertation

Dogan E*****, **Lang P***, Christie MD, Weigel D. The transmembrane protein RST1 affects AGO1 small RNA association.

Achkar NP, Ré DA*, **Lang P***, Stegmeyer G, Milone D, Manavella PA. Environmental conditions switch alternative microRNA biogenesis pathways.

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1. Introduction

From short-lived fruit flies all the way to giant sequoias that are considered to be among the world's largest and oldest living beings, life always starts with a single cell. For the differentiation and specification of cell types, tissues, organs and finally highly complex life forms, cells divide and grow in elaborate patterns. In order for this to finally yield living organisms, every developmental step is tightly regulated in both time and space. One of the major forces that steer these processes is the abundance of gene products, meticulously patterned throughout an organism. Additionally, its fine-tuning is thought to be an important factor in the evolution of complex organisms (for example reviewed in (López-Maury, Marguerat, and Bähler 2008)). Following this hypothesis, one can assume that it is mutation of regulators within a specific gene's expression network rather than merely mutation of the gene itself that drives changes within evolution.

The levels at which gene products are present, as well as their spatial distribution, greatly depends on the cellular, developmental and environmental context. Different endogenous and exogenous factors shape the transcriptional output of a cell's genome. The abundance of a gene transcript is often controlled, both transcriptionally and post-transcriptionally, by a specific group of RNAs, so-called small RNAs (sRNAs).

A large portion of the plant transcriptome is made up of these 20 to 24 nucleotide long regulatory RNAs. While results of their action, namely the silencing of genes through sequence complementary nucleic acids, were already observed and molecularly studied from the late 1980 onwards in the context of transgene-mediated silencing (reviewed in (Prins et al. 2008)), the first endogenous small RNA, identified in the model organism *Caenorhabditis elegans*, was only found more than a decade later (R. C. Lee, Feinbaum, and Ambros 1993). At that time the exact functional principle was unclear, but Lee and colleagues proposed that the detected 22 nt *lin-4* sRNA downregulates the levels of the *LIN-14* transcript through a direct RNA-RNA interaction in a "novel kind of antisense translational control mechanism" (R. C. Lee, Feinbaum, and Ambros 1993). Mutants of *lin-14* have pronounced developmental phenotypes, thus supporting the role of sRNAs in modeling evolution.

Over the years, the number of known types of sRNAs has kept increasing steadily. While regulation of gene expression through various modes of action like chromatin modification, mRNA degradation or inhibition of translation is common to all of them, their origin and molecular characteristics help sorting the different sRNAs into several classes (Nicolas G. Bologna and Voinnet 2014; Axtell 2013).

1.1 Plant small RNAs

The very first observations of sRNA action - although, at the time, not identified as such - were made in plants (A. R. van der Krol et al. 1990; Alexander R. van der Krol et al. 1988; Napoli, Lemieux, and Jorgensen 1990). Napoli, van der Krol and colleagues found that expression of a transgene could inhibit the expression of an endogenous gene with a closely related sequence. Trying to overexpress a chalcone synthase (CHS) for a deeper purple color in petunia, they saw that their approach had the opposite effect, and instead produced completely white or purple-white patterned flowers. They termed the phenomenon 'co-suppression' (A. R. van der Krol et al. 1990; Napoli, Lemieux, and Jorgensen 1990). The corresponding small non-coding RNAs, small-interfering RNAs, that mediate this co-suppression, were described later, six years after the first sRNAs - in this case microRNAs - officially entered the stage in *C. elegans* (R. C. Lee, Feinbaum, and Ambros 1993; Hamilton and Baulcombe 1999). The first systematic isolation and description of plant sRNAs came about only in the beginning of the new century (Llave, Kasschau, et al. 2002; Reinhart et al. 2002). Today, we are aware of a plethora of plant (and animal) sRNA classes that keep growing continuously.

The biogenesis pathways and modes of action of sRNAs are very diverse. Aiming to classify the sRNAs, they were thus subdivided based on different characteristics, for example their template, size or mode of action (Nicolas G. Bologna and Voinnet 2014; Axtell 2013). Owing to the focus of my work on biogenesis, I will here classify sRNAs, based on their origin, into two main types: those that are derived from a single strand of RNA that folds back onto itself into a usually imperfectly paired, 'bulgy' hairpin-like structure (hpRNAs), and the sRNAs that are synthesized from perfectly double-stranded RNA (dsRNA) precursors, called small interfering RNAs (siRNAs) (Axtell 2013). Partly due to different sets of proteins involved in their biogenesis and to very specific sRNA strand

size distributions, siRNAs can be subdivided further, as explained in the following paragraphs (Figure 1, based on (Axtell 2013)).

Even though biogenesis of all siRNAs starts with the formation of a dsRNA precursor, further processing steps and later siRNA activity differ substantially across the different classes. For **secondary siRNAs**, overall biogenesis depends on the activity of an initial sRNA that binds to or causes cleavage of a targeted RNA. Depending on the length and structure of these sRNAs, the specialized RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and a number of cofactors such as SUPPRESSOR OF GENE SILENCING (SGS3) are recruited to convert the cleaved mRNA transcripts into double-stranded RNA (Manavella, Koenig, and Weigel 2012; Peragine et al. 2004; Borges and Martienssen 2015). Further processing, i.e. 'dicing' of the entire length of the dsRNAs by DICER-LIKE 2 or 4 gives rise to secondary 22 and 21 nucleotide long siRNAs, respectively (Z. Xie et al. 2005). If their precursor has a uniformly defined terminus, successive dicing of the dsRNA results in so-called phased siRNAs. While secondary siRNAs normally target mRNAs based on perfect complementarity - thus also their own mRNA origin - they can also act in trans, directing repression of distinct mRNA targets and are thus termed 'trans-acting' siRNAs, or ta-siRNAs (Vazquez, Vaucheret, et al. 2004; Allen et al. 2005). Due to these characteristics, and if the encoding mRNAs are similar enough, a very limited number of mRNAs and corresponding ta-siRNAs can repress many members of a gene family, as it has been shown for the plant *NUCLEOTIDE-BINDING SITE-LEUCINE-RICH REPEAT* (*NB-LRR*) defense gene superfamily (Zhai et al. 2011). Action of these 21- to 22 nt siRNAs is mostly mediated by members of the ARGONAUTE 2/3/7 clade in *A. thaliana*. Through AGO2, they can mediate de novo DNA methylation and play a role in pathogen defense (Harvey et al. 2011; X. Zhang et al. 2011). ZIPPY, also known as AGO7, is in this context involved in both phase transition and adaxial-abaxial patterning, solely through association with the ta-siRNA biogenesis associated miR390 (Montgomery et al. 2008; Husbands et al. 2009).

Different from secondary siRNAs, the mostly 23-24 nt long **heterochromatic siRNAs** (het-siRNAs) typically correspond to intergenic and repetitive genomic regions. For their biogenesis, they largely depend on the alternative DNA-dependent RNA polymerase Pol IV (Mosher et al. 2008), as well as on dsRNA complementation by RDR2, and DICER-LIKE 3 (DCL3) directed dicing (Lu et al. 2006; Llave, Kasschau, et al. 2002). Instead of directly acting on specific mRNAs, het-siRNAs are associated with *de novo*

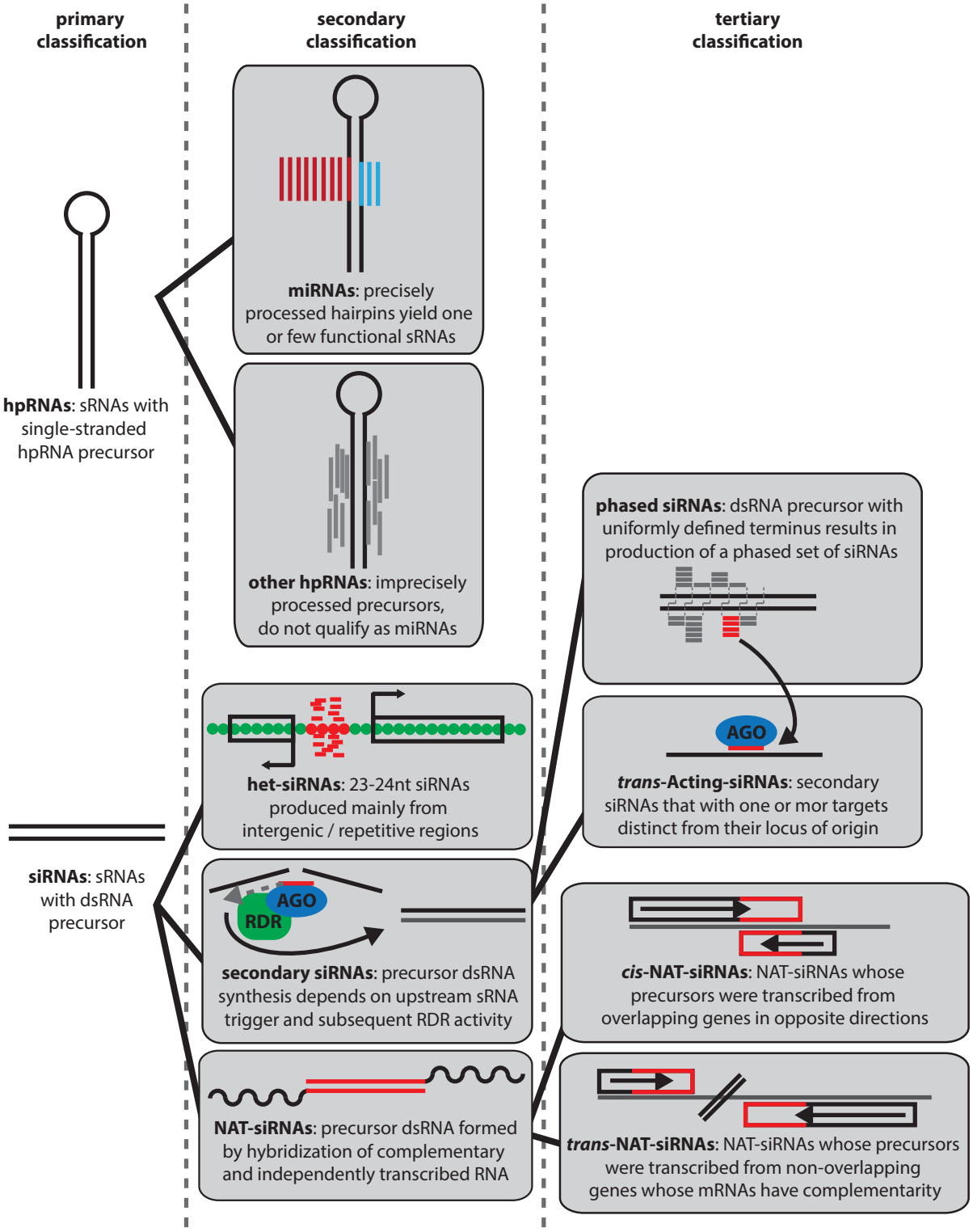

Figure 1. *Classification of small RNAs, modified after Axtell 2013.*

deposition of repressive chromatin modifications at their target DNA (M. Matzke et al. 2009), executed with the help of proteins of the ARGONAUTE 4 (AGO4) clade that

consists of AGO4, 6, 8 and AGO9 (Havecker et al. 2010). Even though het-siRNAs, or Pol IV-dependent siRNAs (p4-siRNAs), are among the most abundant sRNA types in angiosperms, their loci appear to be largely evolutionarily transient even between closely related species (Ma, Coruh, and Axtell 2010). In conjunction with AGO4, they guide methylation within the canonical RNA-directed DNA methylation (RdDM) pathway, causing transcriptional silencing typically at transposon and other repetitive sites (M. A. Matzke and Mosher 2014). AGO4 is, in this context, also involved in methylation-mediated plant defense against DNA viruses, and is a major effector of co-transcriptional gene silencing (TGS).

While both secondary siRNAs and het-siRNAs arise from single sRNAs that function as templates for dsRNA synthesis by an RDR protein, **NAT-siRNA** dsRNA precursors are formed by hybridization of two complementary RNAs that originate from independent transcription events at opposite strands of the same locus (cis-NAT-siRNAs). Stress- or developmentally controlled expression of the antisense transcript can post-transcriptionally regulate the abundance of the corresponding, partially overlapping sense-transcript (Katiyar-Agarwal et al. 2006; Borsani et al. 2005). The possibility of trans-NAT-siRNAs, e.g. NAT-siRNAs that are derived from transcripts originating from non-overlapping genes, remains hypothetical for the moment. Even the general importance of NAT-siRNAs as a class itself is unclear, as presence of the cis-NAT gene configuration does not necessarily imply actual production of sRNAs, and the biogenesis pathways for known NAT-siRNAs are highly diverse and do not give a uniform general picture (Axtell 2013).

SiRNAs make up a large, if not the largest portion in the body of a plant's sRNA complement and play important roles in, for example, the response to biotic and abiotic stresses. In addition to plant endogenous siRNAs that are produced from a plant's own dsRNAs, they can even arise as a reaction to pathogen 'attacks', being produced from exogenous viral RNAs as part of the plant defense response (for example (Csorba, Kontra, and Burgyán 2015)).

1.2 miRNAs and their biogenesis

MicroRNAs are among the most abundant components of both plant and animal sRNA populations, and important factors in post-transcriptional regulation of protein-coding gene expression in higher eukaryotes (Ma, Coruh, and Axtell 2010). Since their discovery in 1993 (R. C. Lee, Feinbaum, and Ambros 1993), an immense body of research has built up, showing that miRNAs play essential roles in development and life in a profusion of contexts, from heart development or diseases like cancer, to leaf formation or the transition from vegetative to reproductive phase in plants. As regulatory components of gene expression, miRNAs are crucial for responses to environmental changes and adaptive development (reviewed in (B. Zhang 2015; Sunkar, Li, and Jagadeeswaran 2012)).

A number of plant miRNAs are highly conserved over a long evolutionary distance, all the way from *Embryophyta* to the core rosids. The majority however is either restricted to closely related species, as it is the case between the *Arabidopsis thaliana* and *Arabidopsis lyrata* or *Capsella* species within the *Brassicaceae*, or even solely specific for one single species. Additionally, new miRNA precursors can arise in a rather straightforward way from self-complementary DNA-sections, inverted repeats or random transcript foldbacks (Felippes et al. 2008; Fahlgren et al. 2007). This suggests an over-representation of young, recently 'born' miRNA genes within plant genomes, with relatively rapid evolutionary turnover compared to a rather low number of older, conserved miRNA families, and thus substantiates a role of miRNAs in tuning expression networks in the context of evolution ((Smith et al. 2015), reviewed in (Cuperus, Fahlgren, and Carrington 2011)).

By definition, plant sRNAs are classified as miRNAs essentially based on the single criterion that they are the ~21 nt long product of precise excision from the stem of a single-stranded hpRNA precursor (Meyers et al. 2008). In plants, their biogenesis mostly starts with the RNA polymerase 2 (RNAPII) mediated transcription of the primary miRNA (pri-miR) from discrete transcriptional units within intergenic regions. Like any other transcript, pri-miRs are subject to subsequent capping, polyadenylation and, in some cases, alternative splicing (M. Xie, Zhang, and Yu 2015; Voinnet 2009).

Cofactors activating miRNA transcription are for example the MEDIATOR complex and the transcription factor NEGATIVE ON TATA LESS 2 (NOT2), the latter being a direct interactor of RNAPII for regulation of *MIRNA* genes (Figure 2, (Y. J. Kim et al. 2011; L. Wang et al. 2013)). Additionally, a number of transcription factors play an important role in miRNA expression and biogenesis. They bind either to *MIRNA* genes or directly regulate the expression of core biogenesis components, such as CELL DIVISION CYCLE 5 (CDC5), which promotes *MIRNA* transcription via promoter association, or the DCL1-transcription affecting XAP5 CIRCADIAN TIMEKEEPER (XCT) (Sun et al. 2015; Fang, Shi, et al. 2015). Additionally, the generic pri-mRNA splicing factors and cap-binding complex proteins CAP-BINDING PROTEIN 20 (CBP20), CBP80/ABH1 and the C2H2-zinc-finger protein SERRATE (SE) are associated with pri-miRNAs and involved in their processing (S. Kim et al. 2008; Laubinger et al. 2008). At least one of the CBPs, CBP20, interacts with SE and NOT2b, further linking the CBC with the processing machinery (L. Wang et al. 2013).

Due to specific base-pairing, the resulting single-stranded pri-miRNA molecule imperfectly folds back on itself, forming the miRNA characteristic structure of loop and imperfectly double-stranded stem, the so-called hairpin. This secondary structure is less distinctive in plants than in animals, specifically as their precursor length is more variable (Reinhart et al. 2002; Llave, Kasschau, et al. 2002), but at least distinctive enough that it, in concert with additional criteria, is used to predict new miRNAs and their potential targets - so far, over 400 miRNAs and around 2000 targets for *Arabidopsis thaliana*, in comparison to over 2500 mature miRNAs and almost one million targets for *Homo sapiens* (miRbase release 21, (Jones-Rhoades and Bartel 2004; Adai et al. 2005; Bonnet et al. 2004; X.-J. Wang et al. 2004; J. Meng, Shi, and Luan 2014; Wong and Wang 2015)).

Structural features in the hairpin determine the two precise sites of cleavage which in plants is exclusively executed by the nuclear RNAse-type III DICER-LIKE1 (DCL1) enzyme, a canonical core component of the processing machinery (Bernstein et al. 2001; Kurihara and Watanabe 2004). In animals, the first cut, that is the pri- to precursor-miRNA conversion, is executed by the nuclear RNAse-type II protein DROSHA, while the second cleavage, now in the cytoplasm, is mediated by DICER (Narry Kim, Han, and Siomi 2009). Both in plants and animals, the location of the first cut within the primary hairpin depends on the highly conserved junction between its single- and double-stranded RNA part. It takes place roughly 11 bp away from this site (Werner et al. 2010; L. Song, Axtell,

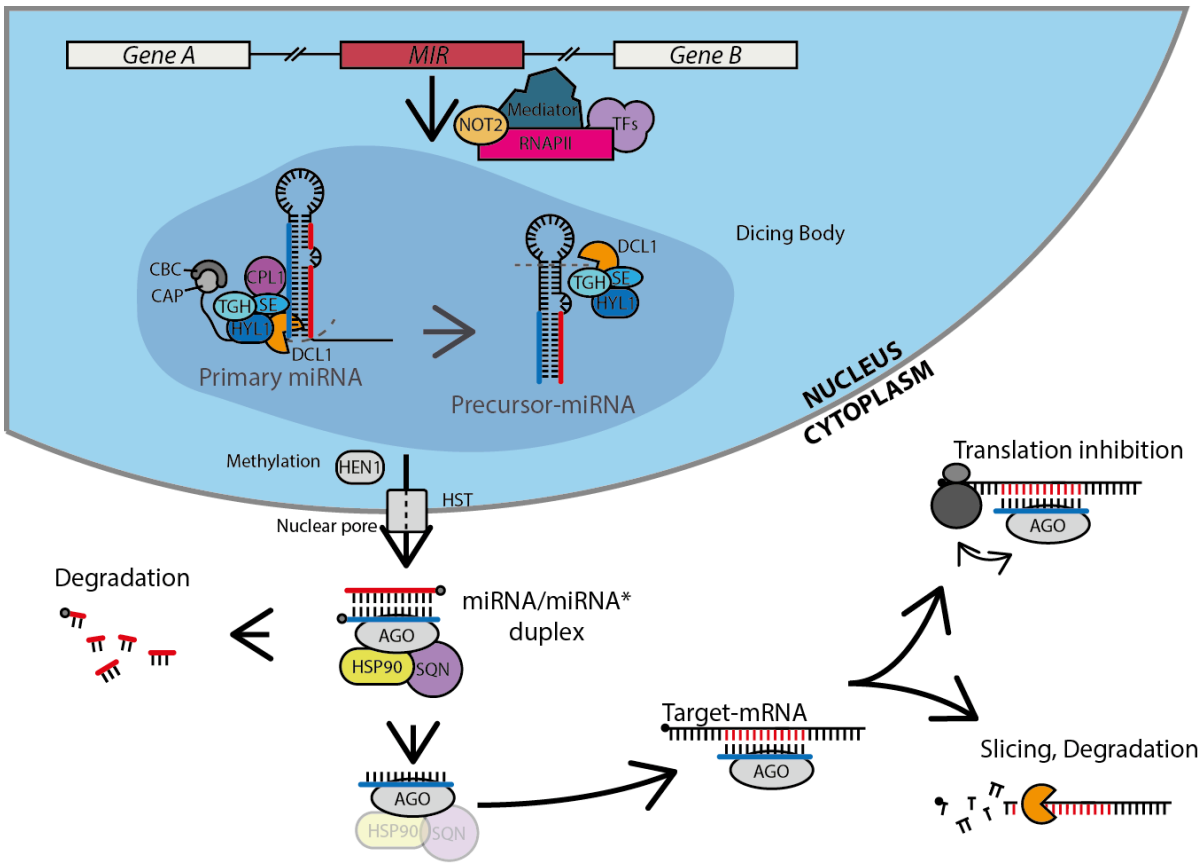

Figure 2. *Simplified schematics of miRNA biogenesis and action.*

and Fedoroff 2010; Mateos et al. 2010; Narry Kim, Han, and Siomi 2009). The 'ruler' length within the DICER enzymes, which is defined by their protein structure, then determines the position of the subsequent second cut, and the final miRNA/miRNA* duplex is generated (Nicolás G. Bologna, Schapire, and Palatnik 2013; Nicolás G. Bologna et al. 2013; Macrae et al. 2006).

DCL1-mediated cutting is carried out in nuclear dicing bodies (D-bodies) known as the processing complex (Figure 2, (L. Song et al. 2007)): Firstly, the nuclear RNA-binding protein MODIFIER OF SNC1 2 (MOS2) helps with recruitment of the pri-miR to the processing complex (X. Wu et al. 2013). DAWDLE (DDL), a forkhead-associated-domain protein and direct DCL1-interactor, is also thought to facilitate the access or recognition of pri-miRNAs by DCL1 through binding to pri-miRNAs, and to stabilize them (B. Yu et al. 2008). In addition to DCL1, main components of the processing complex are the double-stranded RNA-binding protein HYPONASTIC LEAVES 1 (HYL1) (Vazquez, Gasciolli, et al.

2004; Kurihara, Takashi, and Watanabe 2006) that binds to the dsRNA-part of the pri-miRNA, and SE, which connects with the pri-miRNA's ssRNA/dsRNA junction (Lobbes et al. 2006; L. Yang et al. 2006). Both improve the efficiency and precision of DCL1 action through protein-protein interaction. Furthermore, the RNA-binding protein TOUGH (TGH) associates with all main complex-components within the D-body, that is DCL1, HYL1 and SE, contributing to the pri-miRNA-HYL1 interaction and potentially promoting pri-miRNA recruitment to the complex, or alternatively DCL1 cleavage efficiency (Ren et al. 2012). TGH stays associated through both dicing steps, as it binds pri-miRNAs as well as their processing products, the precursor-miRNAs (pre-miRNA). Finally, two direct interactors of SE, namely the RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1) and HYL1-phosphatase C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 (CPL1), are part of the complex during the first cutting steps and play a role in pri-miRNA processing and mediating HYL1 activity, respectively (Speth et al. 2013; Manavella et al. 2012). CPL1 additionally is involved in several aspects of RNA metabolism, as it dephosphorylates RNAPII, contributes to pre-mRNA splicing, appears to be necessary for nonsense-mediated mRNA decay and is suggested to inhibit transcription (Koiwa et al. 2004; T. Chen et al. 2013; Jiang et al. 2013; Cui et al. 2016).

After DCL1-mediated processing, the multidomain AdoMet-dependent small RNA 2'-O-methyltransferase HUA ENHANCER 1 (HEN1) associates with the DCL1-HYL1-miRNA complex and methylates the 3'-termini of the mature miRNA/miRNA* duplexes (Baranauskė et al. 2015; J. Li et al. 2005; B. Yu et al. 2005). This 2'-O-methylation protects the mature miRNA strands from 3' nucleases or HEN1 SUPPRESSOR- (HESO1) mediated 3'-end uridylation and subsequent degradation (Ren, Chen, and Yu 2012; Zhao et al. 2012). Because mature miRNAs mostly, if not exclusively, function in the cytoplasm, they have to be transported out of the nucleus. Detailed mechanisms and the factors in this process have remained unclear, but genetic evidence suggests involvement of the putative miRNA export HASTY (HST), a plant exportin-5 receptor homolog (Park et al. 2005). Once in the cytoplasm, the duplex associates with an ARGONAUTE (AGO) protein, of which *A. thaliana* has 10, typically the ubiquitously expressed AGO1, to form an active RNA induced silencing complex (RISC). Additionally, the more restricted AGO10 is known to associate with miRNAs, specifically miR165/166 (Zhu et al. 2011). Both AGOs are in their

roles distinctly separated due to different expression patterns and developmental functions.

Usually, asymmetric thermostability defines the fate of the two miRNA duplex strands: The strand with the lower 5' stability is preferentially retained by the RISC to mediate target cleaving in its function as the guide strand - formerly also termed the miR strand. Meanwhile, the more stable remaining passenger - mainly the miR*-strand - is degraded (N. Baumberger and Baulcombe 2005; Eamens et al. 2009).

1.3 RISCy business - function of AGOs and their targets

The ARGONAUTE (AGO) family was first discovered in *Arabidopsis thaliana* and named after the tubular leaves of the characteristic *ago1* mutant phenotype, which reminded the authors of little squids (scientific name *Argonauta*, (Bohmert et al. 1998)). The AGOs are conserved across the eukaryotic lineage, but their number greatly varies in different species and even increased during evolution in the plant kingdom. While there is only one AGO copy in fission yeast (*Schizosaccharomyces pombe*), moss (*Physcomitrella patens*) already encodes six, and rice (*Oryza sativa*) even 19. The ten *Arabidopsis* AGOs define three major phylogenetic clades: AGO1/5/10, AGO2/3/7 and AGO4/6/8/9 (reviewed in (Fang and Qi 2016; Nicolas G. Bologna and Voinnet 2014)). Via association with sRNAs and formation of a RISC, AGOs and AGO-like proteins are the main RNA silencing effectors in eukaryotes, for example via targeting of mRNAs through the sequence complementarity of their guiding sRNA. It is thus the AGOs' specific biochemical activity that enables the sRNAs to exert their regulatory functions.

All eukaryotic AGOs consist of four canonical domains: the highly conserved PAZ (PIWI-ARGONAUTE-ZWILLE), the MID (middle) and the PIWI domain as well as a more variable N-terminus (N). While the N-terminal domain's function is yet to be solved, function of the conserved domains in RNAi is rather well understood. Together, the MID and PAZ domain are responsible for binding the single-stranded sRNAs. They specifically recognize and associate with the sRNAs' 5'- and 3'-nucleotides, respectively. Meanwhile, the PIWI domain adopts an RNase H-like fold which conveys endonuclease - i.e. slicing - activity to many AGOs (reviewed in (J.-J. Song and Joshua-Tor 2006; Fang and Qi 2016)).

The bias in the sorting of each sRNA to a specific AGO protein seems to be conferred by the sRNAs length and by its 5' terminal nucleotide. AGO1 and AGO4 predominantly associate with 21 and 24 nucleotide long sRNAs respectively, AGO2 is specialized solely in 21 nt molecules and AGO5 broadly binds the 21, 22 and 24 nt sRNA classes (Mi et al. 2008; Qi et al. 2006). In addition, the AGO proteins have preferences for the 5' terminal nucleotide of the sRNA that they associate with.

Recognition of the sRNAs' 5' terminal nucleotide is mediated by the MID-domain: AGO1 prefers 5' uracil (U), AGO5 cytosine (C) and both AGO2 and AGO4, the latter slightly less pronounced, favor adenosine (A). Considering specific sRNA-subsets, this translates into AGO1 preferring miRNAs, as especially the evolutionarily conserved ones but also most others start with uracil. AGO4 is attracted to hc-siRNA repetitive sequences. Both AGO2 and AGO5 can associate with virus-derived sRNAs, and AGO5 alone finally connects mainly with intergenic sequence-derived sRNAs (Mi et al. 2008; Takeda et al. 2008; Rajagopalan et al. 2006). Further criteria such as duplex-structures, mismatches and different sRNA lengths play an important role in AGO-sorting, as for example both AGO2 and AGO4 have the same 5'-preference, but still manage to largely bind dissimilar sRNAs. Considering that AGO5 is very specifically expressed in parts of the female gametophyte, even the expression patterns of both the sRNAs and the effector proteins have an influence on the partnering of AGOs and their sRNAs (Tucker et al. 2012).

Although plant AGO proteins are, as a result of their potential endonuclease activity, mostly seen as slicer proteins, the modes of action exhibited by AGO/sRNA complexes differ substantially. As mentioned earlier, common to most AGOs is that their action in one way or another results in the repression of specific targets, through repressive chromatin modifications, decline of RNA stability or a diminished efficiency of protein translation. These repeatedly overlapping functions of AGO proteins from different clades demonstrate that their phylogenetic organization not necessarily corresponds to functional separation.

Key ARGONAUTE within the miRNA context as well as for the production and activity of ta-siRNAs is AGO1. This peripheral membrane protein is crucial for the action of endogenous miRNAs on their mRNA targets, inducing both slicing and translational repression (Brodersen et al. 2012). At the same time, it also associates with exogenous,

virus-derived siRNAs and thus plays an important role in resistance and plant defense via antiviral silencing (reviewed in (Fang and Qi 2016; Nicolas G. Bologna and Voinnet 2014)).

Primary step of AGO1-mediated target repression is its association with an sRNA guide strand to form the active RNA induced silencing complex (RISC, Figure 2). In an intermediate step, AGO1 binds the sRNA duplex and, with the help of the chaperone HSP90, associates with CYCLOPHILIN 40 (also known as SQUINT) - a transient interaction that is necessary for successful assembly of a functional RISC (Iki et al. 2012; K. Earley et al. 2010; K. W. Earley and Poethig 2011). Correct orientation of the sRNA duplex in its AGO1 binding is crucial, as this structurally determines which strand is kept (as guide strand) and which one is dismissed from the RISC. Binding of HYL1 to the more thermodynamically stable end of the duplex helps aligning the latter in the correct position for subsequent AGO1-mediated removal of the passenger strand (Eamens et al. 2009).

Through sequence complementarity of the miRNA to its mRNA target, silencing is achieved. Unlike animal miRNA-target-pairs, all so far verified plant miRNAs and their respective targets are extensively complementary to each other (Rhoades et al. 2002). For the canonical plant miRNA-mRNA pairing, this extensive complementarity is crucial in the critical region between the 5' 2nd and 13th nucleotide of the miRNA. Only single mismatches are allowed here, but occur rather rarely. Prediction of target mRNAs is thus straight-forward in plants, as the number of mRNAs that can be bound is limited by the complementarity-requirement. Contrarily, in animals, complementarity to the 'seed' region, i.e. 5' nucleotides 2 to 7, is generally sufficient to predict targets. This minimal requirement results in high numbers of mRNA targets for every miRNA (Bartel 2009). The final RISC action on an associated mRNA however is neither in plants nor in animals regulated by the degree of complementarity, but by additional factors like the cell- or tissue-type, and which proteins are associated with these factors.

Cleavage of the target mRNA, the main effect of miRNA-mediated target regulation, is executed between positions 10 and 11 of the aligned miRNA through AGO endonucleolytic activity (Llave, Xie, et al. 2002). RISC activity can not only affect transcription of targets, but also inhibit translation of mRNA targets via its binding (Brodersen et al. 2008; Gandikota et al. 2007). These silencing effects, whose main effector is AGO1, can be genetically uncoupled, and repression of translation is even

proposed to be the default mechanism of miRNA action (Brodersen et al. 2008; Iwakawa and Tomari 2013). Repression of miRNA target translation happens at the endoplasmatic reticulum (ER), where AGO1 and miRNAs are found in association with polysomes. It requires the integral, ER- and AGO1 associated membrane protein ALTERED MERISTEM PROGRAM 1 (AMP1), which together with miRNAs specifically inhibits target mRNA translation, while it does not have an effect on general protein synthesis (S. Li et al. 2013; Lanet et al. 2009). Depending on the location of the miRNA target sites within the mRNA, the AGO1-RISC can either sterically block the recruitment or movement of ribosomes, or directly confer repression of translation initiation (Iwakawa and Tomari 2013).

Still, AGO1-slicing of transcripts is likely the indispensable, major characteristic of AGO-interacting miRNAs, as a slicer-defective AGO1 is unable to complement mutant phenotypes of *ago1* and slicer-activity appears to be required for effects of most miRNAs on their targets (Carbonell et al. 2012; Arribas-Hernández, Kielpinski, and Brodersen 2016).

1.4 The miRNA Matryoshka - biogenesis regulation within the regulation

Already this very general framework of miRNA biogenesis is sufficient to give an idea of the high complexity of the pathway. As there is such a variety of different factors and cofactors involved in the biogenesis steps and the function of miRNAs, there are naturally at least as many sites that could be targeted for differential regulation. This is likely also necessary to ensure precise patterning of miRNA abundance, or tissue specificity of miRNAs and miRNA machinery, for example in the case of AGO7. Only when the latter is expressed in the cytoplasm and in membranous siRNA bodies, it can, in complex with miR390, trigger the formation of ta-siRNAs from the *TAS3* precursor (Jouannet et al. 2012). Multilayered regulation targeting different parts of the pathway also permits fast, flexible responses to developmental or environmental cues. Modulation of miRNA abundance through the general processing pathway, of a whole miRNA family or in a specific cell, is less complex and more efficient than targeting the transcription of a single miRNA precursor at a time. In addition, a number of essential mechanisms need to be particularly robust and therefore are under the control of both miRNA and stabilizing feedback regulation, as is for example described in more detail below for AGO1 and miR168. Finally, this regulatory Matryoshka harmonizes well with the

idea of regulators rather than genes themselves being the targets and driving forces of evolution.

All these additional layers that ultimately enable a dynamic fine-tuning of miRNA target levels in a responsive way are likely crucial for fitness and survival, to allow plants to rapidly adapt to the environmental context. The 'regulation of the regulators' is thus not merely there to render a complex situation more confusing - on the contrary, it confers stability and versatility to the miRNA pathway so the latter can accomplish its full regulatory potential. Three of the most obvious general regulatory targets in the miRNA hierarchy are the transcription and processing of *MIR* genes, the modulation of proteins involved in the pathway, and finally modification of mature miRNAs themselves, and will be described in more detail in the following.

Like for any coding gene, the regulation of miRNA availability can be modulated directly at the **transcriptional level of *MIR* genes**. A prominent example for this is the promotion of *MIR156A* and *MIR156C* transcription under low sugar conditions in dependence of HEXOKINASE1 signaling activity, and the gradual repression of their transcription with increasing age and the correlated larger plant size, higher photosynthetic activity and thus increased sugar levels (L. Yang et al. 2013; S. Yu et al. 2013). Already between tissues or different developmental stages, **expression and processing** efficiency can differ measurably, as is seen for the age-dependent decrease of miR156 expression, which is seen in very different plant species (J.-W. Wang et al. 2011). Furthermore, they can be affected by various types of stress: Exposure to salt, osmotic, cold or heat stress can for example cause up- or downregulation of a specific set of pri-miRNAs, heat stress being the most efficient regulatory cue (Laubinger et al. 2010; Iglesias et al. 2014). For the majority of pri-miRNAs though, these environmental effects on their steady-state levels are small.

However, even if a specific pri-miRNA stays unaffected, the levels of the associated mature miRNA can, due to changes in processing, often strongly respond to an external signal. Accumulation of mature miR159a for example, but not of its precursors, indirectly responds to abiotic stress, as it changes depending on the levels of the phytohormone abscisic acid (ABA) (Reyes and Chua 2007). Other miRNAs are influenced by environmental conditions in a more direct fashion. *MiR395* and *MiR399* for instance display increased expression upon sulfate and phosphate (P_i) starvation,

respectively (Jones-Rhoades and Bartel 2004; Franco-Zorrilla et al. 2007). Additionally, environmental cues do not only provoke completely unrelated miRNAs to distinct reactions - even miRNAs with multiple encoding loci, i.e. miRNA families, can display different tissue-specificity or differential stress responses, as do the members of the miR395 family (Kawashima et al. 2009; X. Yang, Zhang, and Li 2011). In the animal field, some miRNA families (as well as single miRNAs) were found to rely on highly specialized proteins that specifically regulate the processing of only their precursors, for example the let-7 specific LIN-28 in *C. elegans*, a regulatory level that yet has to be uncovered in plants (Vadla et al. 2012).

One step up in the hierarchy, besides the specific regulation of a single miRNA or miRNA family, the entire pathway can be regulated in a more general way, but still tissue-specifically, through targeting of **components of the miRNA machinery**. As a key protein of the processing chain, HYL1 normally ensures precise and efficient DCL1 action and correct strand sorting. This function is so elementary that multiple pathways target HYL1, either to regulate its protein levels or its activity. For one, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), a RING-finger E3 ligase, protects HYL1 from degradation by an unknown protease. COP1 acts in a dark/light dependent way and through its action directly manipulates overall HYL1 protein levels (Cho et al. 2014). Additionally, the activity of HYL1 can be posttranslationally regulated. Normally, HYL1 is present in plants in different phosphorylation states, either as the hyperphosphorylated, less active version or as an active, hypophosphorylated HYL1. Switching to the active HYL1 form via dephosphorylation is mediated by CPL1 phosphatase activity, while inactivation results from MPK3-dependent phosphorylation (Raghuram et al. 2015). *cpl1* mutants that mainly contain the hyperphosphorylated HYL1 display sRNA profiles similar to *hyl1* plants. Presence of adequate levels of the active HYL1-state thus appears essential for accurate miRNA processing and strand selection (Manavella et al. 2012).

A second factor that is indispensable for the pathway and that is subject to multiple regulatory mechanisms is AGO1. Similar to HYL1, the AGO1 slicer is also differentially expressed across tissue types and developmental stages, reaching its expression peak in the actively dividing shoot and root tissues (Winter et al. 2007). Maintenance of stable AGO1 protein levels is critical for plant development, since *ago1*

mutants have a wide range of defects, which is a likely explanation for a multifaceted, complex regulation that involves both coarse- and fine-tuning components. One of the more subtle balancing switches is F-BOX WITH WD-40 2 (FBW2), a negative regulator of AGO1 that destabilizes the protein through an unknown mechanism (K. Earley et al. 2010). Though mutations in *FBW2* suppress many developmental phenotypes of weak *ago1* mutants by increasing AGO1 protein levels, the same mutations in a wild-type background do not have a strong effect (K. Earley et al. 2010).

This lack of phenotypic defects in *fbw2* mutants can be traced back to the broader regulatory context of AGO1 homeostasis. One of its main features is the negative regulation by *miR168* that implies an AGO1-*miR168* feedback loop. Disruption of this balance, for example in the absence of miR168 regulation, strongly impairs plant development, highlighting the overall importance of the AGO1-miR168 regulatory loop (Vaucheret et al. 2004; Vaucheret, Mallory, and Bartel 2006). Due to its central role in miRNA action, AGO1 is also an obvious target for manipulation by plant pathogens, for example through the viral suppressor of RNA silencing (VSR) P0. This F-box protein hijacks a host E3 ubiquitin-protein ligase to mediate AGO1 ubiquitylation, which ultimately leads to AGO1 degradation via the autophagy pathway and thus disrupts the plant antiviral defense mechanism of posttranscriptional gene silencing (PTGS) ((Csorba et al. 2010; Derrien et al. 2012), reviewed in (Carbonell and Carrington 2015; Csorba, Kontra, and Burgyán 2015)).

Finally, apart from this multitude of mechanisms interfering directly with either the early steps of miRNA biogenesis or proteins involved in miRNA processing and function, also **mature miRNAs** are subject to further regulation. One example is the principle for which the term 'target mimicry' was coined: The earlier mentioned phosphate-starvation induced miR399 normally targets the mRNA of PHOSPHATE 2 (PHO2), an E2 ubiquitin conjugase-related protein also involved in phosphate metabolism. There is however an additional family of transcripts, among them the non-coding *INDUCED BY PHOSPHATE STARVATION 1* (*IPS1*), which contains a sequence region complementary to *miR399*. Different from the canonical miRNA-mRNA complementarity, mismatches between *miR399* and the *IPS1*-transcript cause unusual bulge formation - particularly at positions 10-11 of the miRNA that are critical for the miRNA-induced

cleavage of a paired mRNA. These bulges appear to inhibit slicing activity, resulting in effective competition of *IPS1* with canonical miR399 mRNA targets. In this manner, *IPS1* sequesters the miR399 molecules away from their actual targets and marks them for degradation. The end result is that the starvation-responsive miR399 and *IPS1* fine-tune P_i homeostasis and the response to P_i starvation (Franco-Zorrilla et al. 2007).

So far, the *IPS1-miR399* pair is the only well-documented example for regulation of mature miRNA levels via endogenous target mimicry. Computational analyses have suggested that there might be other miRNA target mimics, but the biological consequence has barely been established for any of these (Y. Meng et al. 2012; H.-J. Wu et al. 2013). Still, since this is such a powerful way to suppress the effects of miRNAs, the *IPS*-transcript has been used as a template to engineer target-mimic (MIM) constructs that aim to reduce the activity of entire miRNA families (Todesco et al. 2010; Franco-Zorrilla et al. 2007). Interference with the miRNA pathway at this level often has - depending on the targeted miRNA - global effects on plant development. Many of the MIM-plants thus display severe phenotypes that correlate with those seen in corresponding miRNA knock-out or knock-down lines, or with what is seen when miRNA-resistant mutations are engineered into miRNA-controlled mRNAs. Together with variants of this technology, such as the short tandem target mimics (STTMs) (Reichel et al. 2015; Yan et al. 2012), they are extensively used to study the biological role of miRNAs.

1.5 The hunt for new cofactors and pathway members

Knowledge of the various regulatory elements and levels of the miRNA pathway is not only beneficial for better understanding of its basic framework, but discovery of additional regulators provides us potentially with means to better explore the intricacies of the complex miRNA machinery. For example, while the overall principle of miRNA action follows similar rules across the plant and animal kingdom, the pathway to mature miRNAs differs between the two in a number of aspects. How many exactly is yet to be determined, as there either have not been extensive, systematic quests for proteins related in sequence and/or function, or known homologs of animal miRNA regulators have often not been studied in plants. By now, a large number of animal protein complexes and cofactors involved in the biogenesis and processing of miRNAs have been

identified and thoroughly examined. Thus, both aspects of miRNA function are rather well characterized. In plants on the contrary, there are still many blank spaces to fill. Especially the multifaceted regulation of miRNA biogenesis is not yet understood much beyond its basic principles. Plants containing mutations in genes encoding proteins of the miRNA pathway display a considerable range of different phenotypes: null mutants of the core biogenesis factors *DCL1* and *SE* die as embryos, whereas null mutants of the similarly crucial *HYL1* and *HEN1* are impaired in their development, but still viable and able to reproduce. The implications of this phenotypic variation are to date unclear. Does it merely reflect redundancy between closely related proteins or proteins with similar function, and diverse requirements in RNA silencing pathways? Or is it indicating differential activity of miRNA-related proteins in distinct tissues and during various developmental stages?

To help solving more of the unknowns in the plant miRNA biogenesis equation, a number of different screening assays have been designed (for example (Jauvion, Elmayan, and Vaucheret 2010; W. Wang et al. 2011; Manavella et al. 2012; Brodersen et al. 2008)). Commonly, forward genetic screens use transgenic plants that provide visually obvious readouts of miRNA activity as reporter background, to simplify finding potential candidates: Brodersen and colleagues for instance employed plants expressing a green fluorescent protein (GFP) containing a target site for an endogenous miRNA (Brodersen et al. 2008). Similarly, the screen recently designed in our laboratory is based on the overexpression of a single cassette holding both a firefly luciferase gene (*LUC*) as well as an artificial miRNA (amiRNA) targeting that very same luciferase reporter (Figure 3A, (Manavella et al. 2012)). Luciferase luminescence is thus elevated in mutagenized plants that cannot produce the amiRNA-Luc or properly execute its silencing function. Due to the clear readout, seedlings can be screened for changes in luminescence at a very early age and in a fast, high-throughput fashion.

This luciferase screen, and an additional screen using an artificial mimicry (MIM) construct as reporter line (Figure 3B), are the sources of candidate genes characterized within the frame of this thesis. The screens were established by Pablo A. Manavella and Michael D. Christie, respectively, and I joined their efforts in characterization of the mutants when candidate genes had already been reliably identified. Both screens considerably minimize the costs and drawbacks of the inevitable re-discovery of already

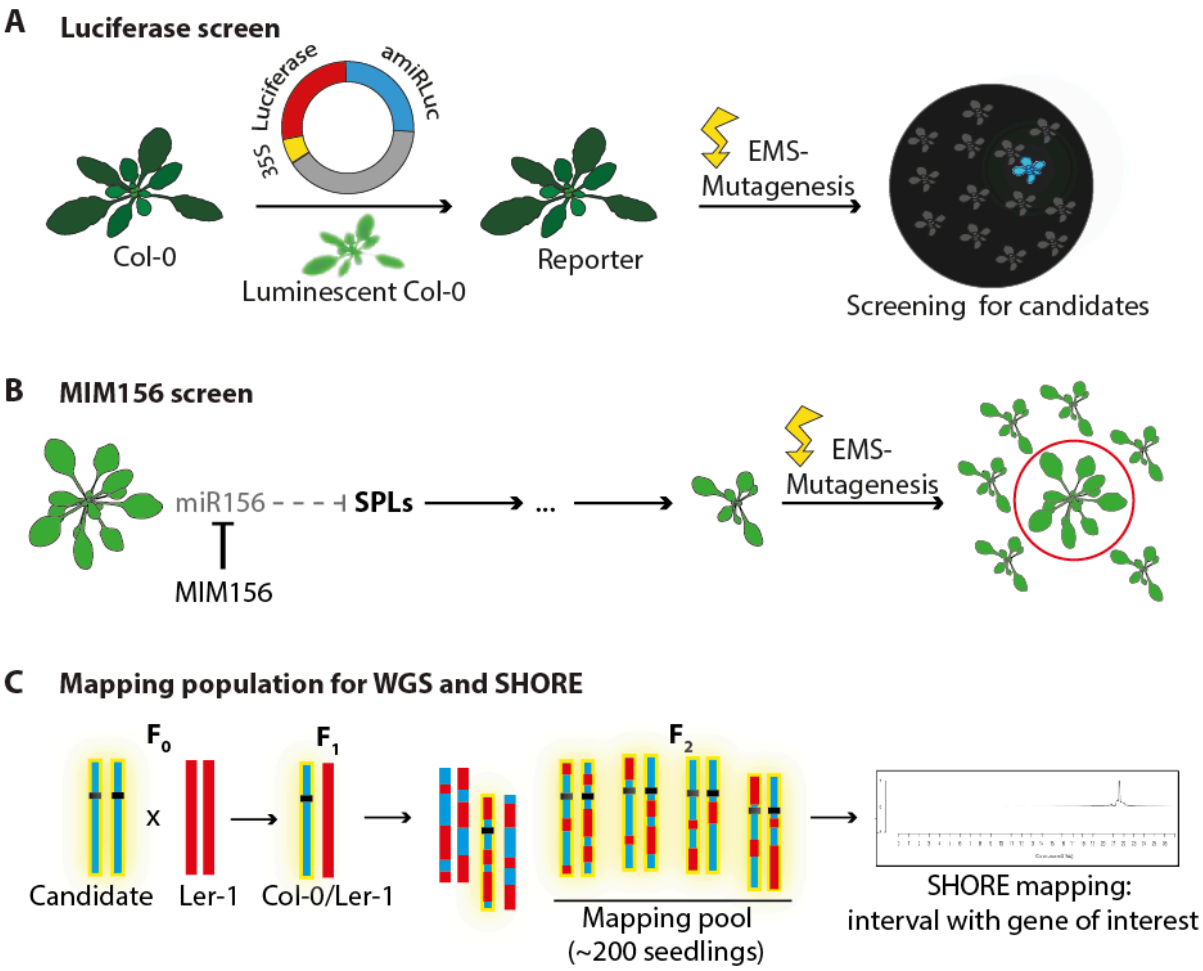

Figure 3. Schematic depiction of screen principles. ***A*** *Wild-type Col-0 plants are transformed with a construct encoding luciferase and an artificial miRNA (amiRLuc) against the Luciferase transcript. Resulting reporter plants are subjected to EMS-mutagenesis, and M2 plants are screened for regain of luciferase activity.* ***B*** *In the mimicry screen, miR156 is sequestered by MIM156, leading to an increase in SPL-transcript levels. The MIM156-reporter is subjected to EMS- mutagenesis and M2 plants are screened for suppression of the MIM156-reporter phenotype.* ***C*** *Candidate plants from a screen are crossed to with Landsberg erecta, mapping pools are created in F2 from plants that are luminescent (Luc-screen) or still suppress the MIM156 phenotype (MIM156 screen), sequenced and results analyzed for an allele frequency bias towards Col-0.*

known miRNA factor genes. They use whole genome Illumina sequencing and SHORE-mapping (Schneeberger et al. 2009) for highly efficient and, most importantly, rapid identification of candidate genes, superseding the additional step of complementation crossing (Figure 3C). In each case, a stable transgenic reporter line, originating from the

Columbia-0 (Col-0) genetic background, was subjected to EMS mutagenesis for identification of new cofactors. Once found within the framework of the screen, mutants from the M_2 generation were crossed with Landsberg *erecta* (L*er*), another fully sequenced *Arabidopsis thaliana* accession. In the F_2 generation, only plants displaying the specific reporter readout were used to create mapping pools for sequencing, as those plants both contain an active reporter construct as well as the homozygous candidate mutation. While the distribution of allele frequencies from the two backgrounds normally is expected to be roughly equal in the F_2 generation, selection for the mutation leads to an allele frequency bias directly at and in the proximity of the causal gene. This bias translates into an above-average presence of genetic information stemming from the - mutated - Col-0 background and a peak in the SHORE allele frequency plot, right at and around the site of the target mutation, enabling identification of the causal candidate gene (Figure S1, (Karlsson et al. 2015)).

As a proof of principle, our luciferase-screen was successfully employed for the identification of new mutant alleles of the main miRNA biogenesis factors *DCL1* and *HYL1*. In addition, a number of intervals were identified that contained genes so far not known to be miRNA-related. Amongst those potential new miRNA biogenesis players was the *HYL1* phosphatase gene *CPL1*, as well as the RNA-processing related *TRANSCRIPTIONAL DEFECTS OF HPR1 BY OVEREXPRESSION 2* (*THO2*) and the tissue-biased *REGULATOR OF CBF GENE EXPRESSION 3* (*RCF3*) (Manavella et al. 2012; Karlsson et al. 2015; Francisco-Mangilet et al. 2015).

Rather than generally influencing the miRNA biogenesis pathway as a whole, the candidates that were found in the Luc-screen are quite specialized for a particular, small functional niche - even though the screen framework they come from very broadly targets miRNA biogenesis as a whole. The second screening approach that is part of this thesis is much more specialized and does not primarily look for miRNA biogenesis, but rather for modifiers of miR156-function in plant development. *MiR156* is a highly conserved miRNA family of eight genes that regulate the abundance of SQUAMOSA PROMOTER-BINDING (SPB) PROTEIN-LIKE (SPL) transcription factors. Among other functions, the *SPL*s are major contributors to phase change between juvenile and adult stage and the control of flowering (Schwab et al. 2005; Gandikota et al. 2007; J.-W. Wang et al. 2008; J.-W. Wang et al. 2011; G. Wu et al. 2009; M. Xu et al. 2016). Sequestration of

miR156 in MIM156 plants causes a characteristic phenotype of shortened juvenile phase, early flowering and spoon-shaped cotyledons (Franco-Zorrilla et al. 2007). In order to find suppressors of this phenotype, the MIM156 transgenic line was subjected to EMS-induced mutagenesis.

In the M_2 population, plants that had lost the MIM156 phenotype were selected as potential candidates (Figure 3B). Identification of the causal genetic lesions again used whole-genome sequencing of a pool of F_2 plants that, after a backcross to L*er*, still showed suppression of the MIM156 phenotype. One of the most promising candidates, *HAWAIIAN SKIRT* (*HWS*), was characterized within the frame of this thesis (manuscript in preparation).

The work presented here adds three new cofactors to different steps of miRNA biogenesis and function. Description of *THO2* in this context further substantiates the connection between miRNA pathways and the general transcription and splicing machinery, and adds detail to our picture of miRNA precursor processing. *RCF3* on the other hand provides a first glimpse of the world of tissue-biased or -specific factors, a regulatory level of miRNA biogenesis and action that is very much understudied in plants. Finally, identification of the F-box protein HWS has the potential to shed light on the principles of target mimicry and could uncover a new connection between the ubiquitination machinery and miRNAs. The candidates characterized here are only a minimal portion of all mutants identified in the frame of the two screening assays conducted in our lab. Many more candidates are still awaiting characterization. In conjunction with newly designed, refined screens targeted at specific parts of the miRNA complexity, they have the capacity to unearth many factors that work together in allowing plants to make full use of the miRNA pathway.

2. Objectives of this work

Since the beginning of small RNA research, plants have been the stage for discoveries of broad impact. The pioneering investigation of virus- and transgene-induced gene silencing paved the way towards our current understanding of gene regulation by small RNAs, and several conserved and central players of this machinery, like the Argonautes, were initially described and studied in plants. A major contributor to this success was the ease and speed of forward genetics in plant model species, particularly *Arabidopsis thaliana*, which is still an excellent system to advance our knowledge of, and discover new players in the miRNA pathway.

In this context, the main aim of my thesis was to shed light on several unknowns within the plant miRNA biogenesis pathway and to fill some blanks with the in-depth characterization of newly found *Arabidopsis* cofactors. Around the start of my work, two large-scale forward genetic screens, broadly aimed at identifying factors involved in miRNA biogenesis, had been developed by my colleagues. Both screens yielded a considerable number of mutants, and after mapping also promising candidate genes. I set out to characterize three of those, all stemming from the two screens described in more detail in part 1.5 of the introduction: *THO2* and *RCF3* from the luciferase-based screen, and the mimicry suppressor *HWS* from the second, mimicry-centered approach.

As a core component of the THO/TREX complex that links transcription and mRNA export, THO2 was already known to be involved in transcription elongation and splicing (Strässer et al. 2002; Rondón, Jimeno, and Aguilera 2010). Additionally, the complex had been suggested to function in RNA silencing and trafficking of siRNA precursors. *THO2* thus was the most promising candidate gene found in the mapping interval (Jauvion, Elmayan, and Vaucheret 2010; Yelina et al. 2010). Due to its involvement in these key regulatory mechanisms, further characterization of *THO2* has improved our understanding of the interconnectedness between transcription, splicing and sRNA pathways.

RCF3, on the other hand, is one of 26 predicted KH domain containing proteins in *Arabidopsis thaliana (Lorković and Barta 2002)*. Owing to its five predicted K homology

(KH) domains - evolutionarily conserved RNA or ssDNA binding motifs - RCF3 is presumably RNA-binding (Siomi et al. 1993). Similar to THO2, a potential connection to miRNAs is already established, as the human KH-type splicing regulatory protein KSRP works in both mRNA decay and in miRNA biogenesis (Gherzi et al. 2010; Ruggiero et al. 2009; Trabucchi et al. 2009). In *Arabidopsis*, RCF3 has been reported to be a negative regulator of heat-stress responsive gene expression and thermotolerance (Guan et al. 2013).

Mutation of *HWS*, found later on to be a MIM-suppressor, causes delayed abscission and partial sepal fusion, which results in the eponymous 'skirt' phenotype (Gonzalez-Carranza et al. 2007). Through its F-box domain and as part of an Skp-Cullin-F-box (SCF) complex, HWS likely participates in ubiquitination and protein degradation, potentially via the 26S proteasome (Ogura et al. 2008; Kuroda et al. 2002). Plants that overexpress HWS are small and display elongated, serrated and highly hyponastic leaves, thereby resembling both *hyl1-2* and hypomorphic *ago1* mutants, which hints at a role in miRNA biogenesis also for this gene (Gonzalez-Carranza et al. 2007).

Further characterization of HWS, THO2, and RCF3 within the framework of miRNA biogenesis is part of this thesis (Francisco-Mangilet et al. 2015; Karlsson et al. 2015).

3. "THO2, a core member of the THO/TREX complex, is required for microRNA production in Arabidopsis"

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Abstract

The multimeric THO/TREX complex plays a conserved role in connecting transcription and nuclear export of mRNAs in both yeast and animals (Strässer et al. 2002). In plants, several members of a complex similar to the metazoan THO2/TREX version have been described so far, including THO1/HPR1/EMU, THO2 and THO3/TEX1 (Furumizu, Tsukaya, and Komeda 2010; Jauvion, Elmayan, and Vaucheret 2010; Yelina et al. 2010). While it is known that mutation of some complex components has effects on siRNA biogenesis (Yelina et al. 2010; Jauvion, Elmayan, and Vaucheret 2010) or alternative splicing of mRNAs encoding serine/arginine-rich proteins (Furumizu, Tsukaya, and Komeda 2010), many other THO/TREX proteins have not yet been functionally characterized. Among these is THO2, essential for both assembly and function of the complex, as null-mutants so far were - unlike those of other THO/TREX components with weaker phenotypes - considered embryo-lethal (Furumizu, Tsukaya, and Komeda 2010; Yelina et al. 2010).

In a forward-genetics screen for cofactors of miRNA biogenesis (Manavella et al. 2012) we identified a new THO2 hypomorphic mutant allele, *tho2-5* and could additionally pinpoint two further alleles, *tho2-6* (knockdown) and *tho2-7* (hypomorphic), from an activation tagging screen and the SALK T-DNA collection, respectively. All three show strong developmental defects in flower architecture, leaves and seeds, resembling plants compromised in miRNA activity and thus reinforcing a potential connection to miRNA biogenesis. Furthermore, our collaborators, led by Anchilie G. Francisco-Mangilet and Soon Ki Park, both then at the Kyungpook National University in Korea, found that the T-

DNA alleles *tho2-1* and *tho2-2* occasionally overcome embryo abortion and, though never surviving to form true leaves, can be used to analyze the THO2 null-mutant phenotype.

As it was known that THO1 and TEX1 are involved in sRNA pathways, we speculated that also THO2 plays a role in this context. Indeed, in the *tho2* background, transgene-induced silencing of a homologous gene is abolished, and levels of secondary siRNAs derived from *TAS1* as well as other endogenous siRNAs are decreased. Additionally, we observed a reduction in the steady-state levels of mature miRNAs and concomitant increase in miRNA precursor molecules and miRNA target mRNAs, a phenotype prompted already by the discovery of *tho2-5* in our miRNA biogenesis screen. However, THO2 appears to locate in different nuclear speckles than HYL1, a core protein of the miR biogenesis machinery, and could not be shown to interact in yeast with any of the 18 tested miR biogenesis-related proteins. We thus hypothesized that THO2 acts in earlier steps common to the different sRNA pathways. Testing various miRNA precursors, we found that all were associated with THO2, and that their interaction with the processing protein HYL1 was reduced in the *tho2-5* background, thus implying a role of THO2 in stabilization of precursor molecules or their transport to the processing machinery.

Motivated by the established connection of THO1 in alternative splicing of genes encoding serine/arginine-rich (SR) proteins, we also sought to demonstrate an association of THO2 with splicing. Of multiple SR genes analyzed, only *SRp34b* displayed differential splicing in *tho2* mutant plants, but we did find a partial overlap in the localization of THO2 and the canonical spliceosome component SRp34. Trying to further pinpoint a potential connection between the observed splicing and sRNA phenotypes, we did not find an effect on splicing of miRNA genes or the transcripts of miRNA biogenesis factors in *tho2*. Also neither transcript nor protein levels of the analyzed miRNA factors were significantly affected. The observed mutant phenotypes appear hence not to be the result of destabilization or differential splicing of miRNA-factor mRNA or problems in translation.

In summary, THO2 appears to be involved in both mRNA splicing and miRNA precursor actions, similar to miRNA-biogenesis cofactors like CBP20 or CBP80, and might interact or act in concert with one of the latter, a hypothesis that needs to be further tested experimentally.

Contributions

Isolation and phenotypic characterization of mutants: AGFM*****, PAM (both Fig. 1, 2, 3, 4)

THO2 expression analysis: AGFM***** (Fig. 5)

Effects of *THO2* mutation on sRNA accumulation: AGFM***** (Fig. 6a-b), PAM (Fig. 6c-d), PK (Fig. 6e-f)

Colocalization of THO2 with miRNA factors: PL (Fig. 7a)

THO2 association with pri-miRNAs: PAM (Fig. 7b-e)

THO2 effect on splicing: AGFM***** (Fig. 7f-g)

miRNA accumulation in *tho2-5* complemented plants: PAM (Fig. S1a), FRK (Fig. S1b)

Y2H assay for testing THO2 interaction with a collection of miRNA-related factors: PK (Fig. S2)

Splicing patterns in *tho2* mutants: AGFM***** (Fig. S3a), PK (Fig. S3b)

Splicing patterns and mRNA accumulation of miRNA biogenesis factors in *tho2* mutants: PK (Fig. S4a-b)

Protein accumulation of miRNA biogenesis factors in *tho2* mutants: PAM (Fig. S4c)

Y2H assay for testing THO2 interaction with DDL, ABH1 and CBP20: PAM (Fig. S5)

***** indicates joint work of AGFM with local collaborators MHK, HJE, SAO, JHK and SKP

See Appendix I

4. “KH domain protein RCF3 is a tissue-biased regulator of the plant miRNA biogenesis cofactor HYL1”

Karlsson P, Christie MD, Seymour DK, Wang H, Wang X, Hagmann J, Kulcheski F, Manavella PA (2015).

PNAS 10.1073/pnas.1512865112

Abstract

MiRNA biogenesis is crucial for posttranscriptional gene regulation in many multicellular organisms. For fine-tuning of the production and levels of miRNAs, a plethora of cofactors is involved in the biogenesis machinery. While null mutants of some plant miRNA factors, like *DICER-LIKE 1* (*DCL1*) and *SERRATE* (*SE*), are lethal, others, among them *HYPONASTIC LEAVES 1* (*HYL1*), *HUA ENHANCER 1* (*HEN1*) or *C-TERMINAL PHOSPHATASE-LIKE 1* (*CPL1*), only produce diverse developmental and physiological phenotypes. The causes of this phenotypic diversity, be they genetic redundancy, divergent requirements for processing of different precursors, or potentially tissue- and stage-specific activity, are still unclear. To fill these gaps in our understanding of the plant miRNA machinery, a number of genetic screens have been developed, looking for further actors in the pathway.

In two independent forward-genetics screens for cofactors of miRNA biogenesis that were performed in our lab, we identified two new mutant alleles of *REGULATOR OF CBF GENE EXPRESSION 3* (*RCF3*), known to be a negative upstream regulator of heat stress-responsive genes and thus thermotolerance (Guan et al. 2013). RCF3 is one of 26 *Arabidopsis thaliana* K-homology (KH) proteins and is predicted to contain five KH domains. These domains contain evolutionarily conserved RNA or ssDNA binding motifs (Siomi et al. 1993). Since the human KH-type splicing regulatory protein KSRP plays a role in both mRNA decay and as key component in Drosha and Dicer microprocessor complexes (Gherzi et al. 2006; Ruggiero et al. 2007; Trabucchi et al. 2009), RCF3 seemed to be a promising candidate for action within the miRNA context.

Several articles on RCF3 function, published during the course of this project, confirmed this notion: This stress regulatory protein, also termed SHINY 1 or HIGH OSMOTIC STRESS GENE EXPRESSION 5, interacts with miRNA-factor CPL1 in the context of transcriptional and co-transcriptional processes, for example modulating mRNA capping and polyadenylation (Jeong et al. 2013; T. Chen et al. 2013; Jiang et al. 2013). Interaction with and dephosphorylation mediated by CPL1 is required for RCF3 subcellular localization, and both RCF3 and CPL1 also interact with the two splicing factors RS40 and RS41 (T. Chen et al. 2013; T. Chen, Cui, and Xiong 2015). All three, RCF3, RS40 and RS41, bind pri-miRNAs, affect the biogenesis of a subset of miRNAs and are required for correct strand selection as well as miRNA level maintenance (T. Chen, Cui, and Xiong 2015).

Using leaf tissue of our mutant alleles, *rcf3-3* and *rcf3-4*, we could only find weak effects on the steady state levels of miRNAs, miRNA targets and miRNA precursors, and no obvious changes in the abundance of miRNAs and miRNA*s. RCF3 expression analysis, looking both at the luciferase activity in reporter lines as well as at *in situ* hybridization and RT-qPCR indicated a strong expression bias towards the vegetative apical region, including young leaves and leaf primordia (from here on referred to as 'vegetative apex'). Consequently, analysis of samples containing mainly vegetative apex showed a clear reduction of miRNA levels in the *rcf3* background, paralleled by an increase in target mRNAs. Similar changes were observed in reproductive apices, indicating that RCF3 affects miRNA accumulation and activity in a tissue-biased way. As was established earlier, RCF3 accumulates in nuclear speckles and thus colocalizes with DCL1, SE and CPL1, factors of the miRNA machinery, further confirming the miRNA biogenesis connection.

Genome-wide analysis of sRNAs in vegetative apices verified a specific reduction of miRNA levels and overaccumulation of miRNA*s, whereas other sRNAs were unaffected by *RCF3* mutations. This phenotype is similar to defects seen in *hyl1* and *cpl1* mutants, and together with the protein-interactions between CPL1, the partly redundant CPL2, HYL1 and RCF3 this could hint at RCF3 acting in miRNA biogenesis through CPL1,2 and possibly HYL1. The latter's phosphorylation depends on CPL1,2 and MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3), and is indeed also affected by RCF3: HYL1 levels in the *rcf3* mutant in young tissue shift from the hypo- towards the less active,

hyperphosphorylated form, a change even stronger than what is observed in *cpl1* background. Furthermore, overexpression of the hypo-, but not the hyperphosphorylated HYL1 suppressed morphological defects of *rcf3-4* leaves and overall morphology, which fits with the *RCF3* expression peak in the vegetative apex where leaf shape is determined.

With our findings, we substantiated RCF3 as a miRNA biogenesis factor, having discovered that it acts preferentially in vegetative and reproductive apices and there promotes HYL1 dephosphorylation, likely through interaction with CPL1,2. Our working model is that DCL1, and possibly SE and CPL1 are among the first components associating with pri-miRNAs. Subsequently, HYL1 and RCF3 - in their active, hypophosphorylated status - are recruited to this complex, promoting strand selection and miRNA processing. How RCF3 acts at the physico-chemical level remains to be elucidated.

Contributions

Identification and phenotypic characterization of *rcf3* mutants: PAM (identification of mutant allele, Fig. S1a), MDC (identification of mutant allele, Fig. 1b, S1a), FK (initial characterization), JH (WGS data analysis), PK (Fig. 1a,c-d, S1b-d)

RCF3 expression and activity via Luc imaging and *in situ* hybridization: PK (Fig. 2)

miRNA levels and activity in *rcf3* mutants: PAM (Fig. 3a-f), HW (Fig. 3g)

Analysis of sRNA sequencing: PK (sample and library preparation), XW (adult leaf data, Fig. S3e), DKS (apex data, Fig.4)

Subcellular localization of RCF3 via confocal imaging and interaction with CPL1 and CPL2 via Y2H: PK (Fig. 5)

Effect of *rcf3* on HYL1 phosphorylation: PK (Fig. 6)

Phylogenetic analysis of RCF3 homologs: PK (Fig. S2)

Effects of *rcf3* on miRNA accumulation and action in leaves: PK (Fig. S3a-d), XW (Fig. S3e)

RCF3 expression and activity: PK (Fig. S4a,c-f), PAM (Fig. S4b), HW

Effects of *rcf3* on pri-miRNAs and miRNA processing factors: PK (Fig. S5)

RCF3 genomic location and promoter activity: PAM (Fig. S6), PK (Fig. S6b)

Screen for testing THO2 interaction with a collection of miRNA-related factors: PK (Fig. S7a), FK

BiFC interaction assay of RCF3 with CPL1 and CPL2: PAM (Fig. S7), PK

In silico expression profiles of *RCF3*, *CPL2*, and *MPK3*: PAM (Fig. S8)

See Appendix II

5 . “A role for the F-box protein HAWAIIAN SKIRT in plant miRNA function”

Lang P*, Christie MD*, Dogan ES, Hagmann J, Weigel D.

(manuscript in preparation)

Abstract

MiRNAs are major contributors to the finely tuned spatio-temporal regulation of gene expression. In order to control accumulation of miRNAs themselves, miRNA genes can be subject to post-transcriptional regulation. One such mechanism is endogenous miRNA target mimicry. It reduces the levels of active miRNAs by sequestering them from their actual target mRNAs, which subsequently leads to an increase of the latters’ levels (Franco-Zorrilla et al. 2007). Based on this principle, transgenic miRNA target mimicry lines (MIMs) have been engineered to knock down *Arabidopsis thaliana* miRNA families for research purposes (Todesco et al. 2010).

One line, MIM156, is a target decoy for members of the highly conserved plant miR156 family, which regulates many members of the SQUAMOSA PROMOTER-BINDING (SPB) PROTEIN-LIKE (SPL) transcription factor family. Repression of miR156 activity causes characteristic spoon-shaped cotyledons and speeds up the transition between the vegetative juvenile and the vegetative adult phase (J.-W. Wang et al. 2008; Schwab et al. 2005). We EMS-mutagenized a MIM156 line, looking for mutations that caused suppression of this very phenotype to find new cofactors within the fields of miRNA biogenesis, function or the regulation of SPLs. Due to the already reduced miRNA levels in the MIM156 background, this screen enables the identification of negative regulators of the miRNA biogenesis or action pathway.

Mutations in the *HAWAIIAN SKIRT* (*HWS*) F-box encoding gene (At3G61590) suppressed the MIM156 defects. Trying to assess if this suppression was specific to miR156, we also tested other MIM lines and found that *hws* can suppress the phenotypes of MIM159, MIM164 and MIM319, indicating that its effect is not limited to the miR156 pathway. Levels of *IPS*, the first identified endogenous MIM, which targets *miR399*, and

of the *miR399* target *PHO2* however are barely changed in the *hws* background, suggesting that HWS is not simply a general miRNA target mimic regulator.

Like the previously published alleles, *hws-1* and *hws-2*, *hws-3* is impaired in the shedding of floral organs, which leads to the eponymous 'skirt' phenotype. Additional *hws* phenotypes include cauline leaf fusions and reduced leaf serrations. Similar organ fusions and changes in leaf serrations are often observed in the context of miRNA related mutants like those of the *CUC* miRNA targets, or of *se* and *abh1* alleles. In agreement with this, overexpression of HWS causes severe developmental abnormalities, reminiscent of mutants like *hyl1-2* and *ago1-27*. Analysis of steady state levels of miRNAs and of miRNA targeted mRNAs in a HWS-overexpressing line revealed a decrease of several miRNAs and corresponding target-mRNA increase, which was mirrored by the inverse observation in *hws*. Together with the broad effect on developmental abnormalities induced by different MIM transgenes, this suggests that HWS could play a more general role in the miRNA pathway, probably upstream of miRNA target stability.

Epistatic genetic interactions of *hws* with miRNA biogenesis factor mutants further supported the idea that both share a common pathway. However, we did not find any direct physical interactions between HWS and known miRNA biogenesis components. HWS does though appear to be associated with ASK1, ASK2 and CUL1 to form an Skp-Cullin-F-box (SCF) complex, which is involved in targeting substrates for ubiquitination. Both suppression of the skirt phenotype in *35S::HWS* as well as the typical overexpressor phenotype are abolished when the transgene lacks the F-box (*35S::mHWS*), indicating that this domain is important also for HWS action within the miRNA biogenesis pathway. Yet, we could not show that HWS E3 ligase activity affects miRNA factors directly, as AGO1, a factor already known to interact with several F-box proteins (K. Earley et al. 2010; Bortolamiol et al. 2007; Nicolas Baumberger et al. 2007), could not be shown to be perturbed in context with 35S::HWS or 35S::mHWS, neither in its protein or mRNA abundance, nor in its ubiquitination status.

Even though our results strongly suggest a connection between HWS action and miRNA biogenesis, it remains unclear how HWS - possibly in its role as an F-box protein - influences the pathway. Finding potential targets of HWS-SCF-complex action and interaction as well as in-depth biochemical analysis of HWS-function, and especially

indirect investigation of its effects via RNA-seq will be crucial for further characterization of HWS within the known miRNA framework.

Contributions

Screen development and *hws* identification and characterization: MDC (Fig. 1) JH (WGS data analysis)

Mimicry suppression phenotype: MDC (Fig. 2a,d), ED (Fig. 2b), PK (Fig. 2c, S2)

Effects of *hws* on miRNA accumulation and action: MDC (Fig. 3a), PK (Fig. 3b-d, S3e-f)

Genetic crosses of *hws* with miRNA related mutants: MDC (Fig. 4a), PK (Fig. 4)

Overexpression of HWS with and without the F-box domain: MDC (Fig. 5a), PK (Fig. 5c)

Extensive phenotyping of *hws* and HWS-overexpressors: PK (Fig. S1a-e, S3C), MDC (S3A)

HWS expression: MDC (Fig. S3D), PK (Fig. S1f)

Screen for testing HWS interaction with a collection of miRNA-related factors: PK (Fig. S4)

Connection of HWS with AGO1: ED (Fig. S5a), PK (Fig. S5b-c)

See Appendix III

6. Discussion

Since the discovery of miRNAs more than 20 years ago, a wealth of cofactors involved in miRNA biogenesis, in the assembly of active RISC complexes, as well as in the faithful repression of target RNAs has been identified and characterized in various model systems. The description of those cofactors has immensely broadened our knowledge of the roles of miRNAs beyond the level of understanding that came from the initial knowledge of core miRNA factors such as HYL1 or SE. We have learned how they can quickly change levels of entire miRNA families, as does the *C. elegans* cell-fate succession and pluripotency-regulating protein LIN-28 (Vadla et al. 2012), or promote the maturation of specific subsets of miRNA precursors, as does the human KH-type splicing regulator KSRP (Trabucchi et al. 2009). Other ancillary miRNA factors act on multiple levels in response to environmental stresses to increase the organism's chance of survival. Prominent example here is p53, a human tumor suppressor that is activated upon DNA damage. In cancer cells, it both induces transcription of a specific miRNA family and enhances processing of additional pri-miRNAs, all of which ultimately decreases the rate of cell proliferation (reviewed in (Hermeking 2007; Leung and Sharp 2010)).

Still, for plants, even though they were the pioneer system for siRNA discovery, many fewer cofactors are known and the details of processing and action are less well understood than in their animal counterparts (Axtell, Westholm, and Lai 2011; Cuperus, Fahlgren, and Carrington 2011). This is partly due to fewer scientists working on basic plant-related research compared to those working on animals and humans, even though plants are powerful study systems for genetic analyses of many essential processes. Different from animals, plants are much more robust to mutations to both inactivation and overexpression of key factors, most likely because their development is so plastic, and even greatly compromised plants can still survive and set (a few) seeds.

In my doctoral research, exploiting the many advantages of plants for the research questions I am interested in, I thus set out to find novel regulatory players on the stage of plant miRNA function. My ultimate goal was to better understand the conserved fine mechanics of miRNA biogenesis and action, and how a plant gets their

spacing, timing and dosage 'just right' to be healthy and happy. Here, I would like to discuss implications of screen design on the type of factors that can be discovered, the functional directions in which the new cofactors that I characterized appear to take us, and how to integrate all these facets into the big picture of plant miRNA biology.

6.1 MiRNA processing - not home alone, and how to screen for its company

Among the first miRNA-related factors to be discovered and characterized in detail were the most essential players of miRNA biogenesis including DCL1, SE and HYL1. Their lack or mis-function causes severe problems in plant growth and development, making them easily detectable in most broadly aimed miRNA screening assays. The role of more recent additions to the miRNA 'team' tends to be more limited, for example to fine-tune other factors' action in the context of developmental age or to catalyze a differential response to environmental conditions (Huo, Wei, and Bradford 2016; S. Zhang, Liu, and Yu 2014). To pinpoint the exact function of a new cog in the wheels of miRNA machinery thus often proves more difficult than it was for the conserved, ubiquitously active factors, as is for example the case for the here presented HWS (manuscript in preparation). It is important to emphasize that it is not necessarily the more limited role of the new factors that complicates their characterization.

Perhaps the most important issue is that factors surfacing now are rarely specialized for the miRNA pathway, but usually have important roles in other biological contexts as well (for example (Francisco-Mangilet et al. 2015)). To decipher their miRNA-related activities, one needs to zoom out and consider these other processes. Especially in the animal field, there is more and more evidence that several pri-miRNA processing steps, including splicing or capping, take place co-transcriptionally (Figure 4, (Bentley 2014)). A number of proteins have been shown to be associated with pri-miRNAs, DROSHA and chromatin at the same time. Multiple miRNA-containing transcripts appear to be cleaved co-transcriptionally by DROSHA, and processing is impaired when nascent pri-miRNAs are prematurely released from chromatin (H. Liu et al. 2016; Morlando et al. 2012; Morlando et al. 2008). Furthermore, both the kinetics and the efficiency of pri-miRNA processing are highly increased in *in vitro* assays when processing is not spatially separated from pri-miRNA transcription by RNAPII (Yin, Yu, and Reed 2015). In plants, the

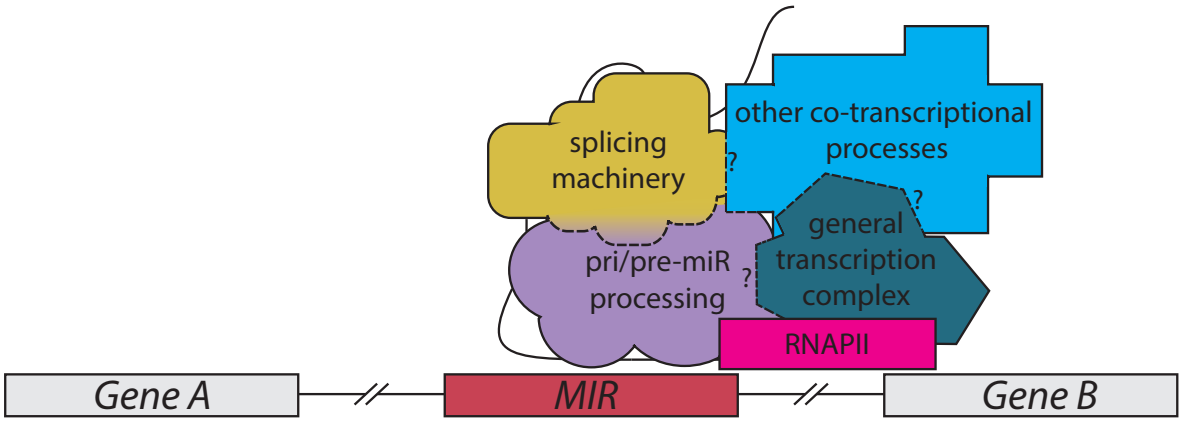

Figure 4. Co-transcriptional processes. *All depicted co-transcriptional processes are carried out by a complex of numerous proteins and transcription factors. Dashed lines indicate overlapping functions of proteins involved in the different processes.*

first direct evidence of co-transcriptional miRNA processing was published just last year, revealing that pri-miRNA transcripts and DCL1 associate with the chromatin of miRNA loci, and that disruption of the transcription elongation factor Elongator complex reduces presence of RNAPII at *MIR* genes, pri-miRNA transcription, and also the association of DCL1 with chromatin (Fang, Cui, et al. 2015).

It thus seems to be merely a question of time until the co-transcriptionality of miRNA processing becomes better understood in plants as well. With this, miRNA processing and the involved factors join the immense crowd of proteins associated with transcription - not only spatially, but also functionally (Figure 4). In this context, it is no longer unexpected that already many factors found through genetics and biochemistry as being important for specific steps of miRNA biology play dual roles, such as CBP20, CBP80 and the *Arabidopsis* pre-mRNA processing factor 6 homolog STABILIZED1 (STA1), working both in miRNA processing and splicing (Laubinger et al. 2008; Ben Chaabane et al. 2013). Furthermore, this increasing 'merging' of molecular pathways can explain how it is many times difficult to disentangle the role of new factors found, as their effects on the miRNA-side, if they do play multiple roles in transcription-related processes, can be both direct and indirect when proteins are simply 'standing in each other's way'.

When designing new genetic screens for the quest of further cofactors to complete the miRNA puzzle, this increasing ambiguity has to be kept in mind. The

development of screens thus faces both the challenge to not only detect what is already known, and to identify players that still have, beyond functions in other co-transcriptional processes, a direct role for miRNA biology. Our broadly aimed luciferase-based screen, origin of the first two miRNA biogenesis cofactors described in this thesis, is highly efficient due to easy, high throughput screening that is possible already at a very early seedling stage, and neither requires lots of space nor special experimental conditions (Manavella et al. 2012; Karlsson et al. 2015; Francisco-Mangilet et al. 2015). Also, the LUC readout is quantitative and thus facilitates the discovery of mutants with very subtle changes in LUC activity and categorize them with respect to the strength of their phenotype. Additionally, in spite of its 'broad' nature, it enables us to even see differential upregulation of the reporter. Identification of two alleles of the tissue-biased *RCF3* in two different, very general *LUC*-based miRNA biogenesis screens in this lab underlines the capacity of our screens to sensitively find also factors with comparatively modest effects on miRNA function. Yet, had the same alleles been identified in a more targeted, organ- or tissue specific screen, the characterization of the mutant genes could probably have advanced at a faster pace.

Some regulatory events are very specific to a given cellular context or developmental timeframe, as is for example the action of RNA-binding LIN28 in animals, which maintains pluripotency in undifferentiated tissues at early developmental stages ((reviewed in (Tsialikas and Romer-Seibert 2015)). Normally, identification of such specialized proteins based on uniformly expressed reporter systems is not trivial and requires laborious and time-consuming analyses of the reporter readout. Organ-specific designs are therefore more adequate approaches. Due to their spatial restrictiveness, they offer the advantage of achieving higher sensitivity than broadly targeted screens, and they facilitate initial characterization, as they already place the candidates into a specific context of action and function. This can help approaching an inconnu when finding genes of 'unknown function', an always present possibility when conducting genetic screens, and be almost as useful as finding a gene or protein that has already been characterized in a different context.

No matter whether organ specific or broadly targeted, genetic screens such as our luciferase-based approach merely report malfunction of miRNA biogenesis and/or downstream processes that have an impact on target regulation, in this case indicated by

a continued lack or regain of luc-mediated luminescence (Manavella et al. 2012). Moreover, novel cofactors that are identified, such as THO2 and RCF3, might play specialized roles, with effects in specific niches of the pathway that are barely detectable outside of the reporter-system context (Karlsson et al. 2015; Francisco-Mangilet et al. 2015). A sensitized reporter background can help unveil factors whose function is masked by prominent essential factors of the pathway. Under normal circumstances, these factors cannot be discovered, as loss of their 'masks' usually is lethal. In our MIM156-based screen, we combine these two aspects: Transgenic MIM156 plants already have reduced activity of miR156 (Todesco et al. 2010), allowing identification also of negative regulators of miR biogenesis. Moreover, screening is fast and efficient, as changes such as suppression of the MIM156-typical phenotype are very easily seen already in young seedlings (Figure 1B).

The power of further screens for the discovery of further cofactors is vast, and only limited by creativity. Which screen design is most promising, apart from the crucial, common sense properties of cost- and time-efficiency to minimize the drawbacks of discovering the usual crowd of miRNA biogenesis factors, or dispensable extras, depends on the aim of a screen. In this work, I characterized candidates stemming from two different types of screens, one broad, the other with a much narrower scope. In either context, characterization of a candidate gene can be complicated and far from straightforward, especially when molecular pathways converge, as it is the case with miRNA processing and general transcription. Or when a reporter background is so sensitive that the mutant alleles discovered, once they are in a 'natural' background, lack a clear phenotype.

Based on the work presented here, I propose two additional points worthwhile of consideration in the context of future genetic screens, both related to the reporter and the phenotype of identified candidates. Independent of the strength of the reporter output, at least one easily distinguishable mutant trait should be the first criterion when evaluating new candidates - visible also outside of the reporter background. It tremendously facilitates follow-up of the mutation in different backgrounds or when doing genetic crosses, and serves as sanity check ensuring the genetic identity of plants when sampling for experiments. Pivotal is also the choice of the reporter itself, as it

might display an unexpected bias. Different screens aimed at various biological contexts, but all employing Luciferase activity as a reporter readout, tend to unearth the same genes (for example, *RCF3*), genes that are often not found in assays targeting the same contexts but using different reporters. What kind of molecular basis this could have, and what this means for the accuracy of a screen, is unclear. It certainly indicates that just by choice of reporter, we are probably unintentionally excluding a number of factors from our setup. And it puts further emphasis on the importance of carefully targeted design that bears in mind the risks and shortcomings of genetic screens.

6.2 *THO2*, splicing and inspiration from the animal field

One of the first genes identified in our luciferase screen that we followed up on was *THO2*, encoding a core component of the multimeric THO/TREX complex, as we had indications from other systems and from homologs in other species pointing towards a *bona fide* role in miRNA function. Also, the THO/TREX complex and its function in linking transcription and mRNA export had already been described thoroughly in yeast and animals, where it is involved in RNA polymerase II-dependent transcription elongation, mRNA splicing and nuclear export of mRNAs (Strässer et al. 2002; Reed and Cheng 2005; Piruat and Aguilera 1998). *THO2* presented thus one of the easier scenarios one can encounter when facing new candidate factors for a biological pathway (Strässer et al. 2002; Peña et al. 2012). Even in plants, a number of complex components were already known and partly characterized, among them THO1/HPR1/EMU and THO3/TEX1 (Furumizu, Tsukaya, and Komeda 2010; Yelina et al. 2010; Jauvion, Elmayan, and Vaucheret 2010). Their involvement in differential splicing and siRNA biogenesis was a good indicator for a potential role of the functionally yet uncharacterized THO2 in an sRNA, and possibly the miRNA, pathway (Furumizu, Tsukaya, and Komeda 2010; Jauvion, Elmayan, and Vaucheret 2010; Yelina et al. 2010). A biological scene of its action was thus set, greatly facilitating further exploration of the specific role of THO2.

The study of *Arabidopsis* THO2 function has been challenging, as the only identified available T-DNA lines, *tho2-1* and *tho2-2*, aborted at the embryonic stage (Furumizu, Tsukaya, and Komeda 2010; Jauvion, Elmayan, and Vaucheret 2010; Yelina et al. 2010). This stalled the characterization of THO2, but at the same time made it a

particularly interesting study target, as the strong phenotype indicates that THO2 likely plays a more important or broader role in development than other components of the THO/TREX complex, a notion that was already advanced earlier (Furumizu, Tsukaya, and Komeda 2010; Jauvion, Elmayan, and Vaucheret 2010; Yelina et al. 2010). Finding that a small portion of homozygous *tho2-1* and *tho2-2* T-DNA mutant plants do survive the embryonal stage, together with the identification of the novel *tho2-5*, *tho2-6* and *tho2-7* alleles, gave us a unique set of tools to finally study THO2 function in plants (Figure 1-5, (Francisco-Mangilet et al. 2015)). Our assortment of mutants is specifically valuable as it combines a broad severity range. It includes both T-DNA mutants that, by their nature, usually are null mutants, and new alleles recovered from an EMS screen where one generally also finds weak and partial loss-of-function alleles.

In concert with results from previous studies of the THO/TREX complex components HPR1 and TEX1 (Furumizu, Tsukaya, and Komeda 2010; Jauvion, Elmayan, and Vaucheret 2010), we found that THO2 affects various sRNA pathways. The *tho2* mutant displays decreased levels of both siRNAs and mature miRNAs, and consequently increased target mRNAs and miRNA precursor molecules (Figure 6, (Francisco-Mangilet et al. 2015)). Trying to narrow down the place of THO2 action, we did not detect direct interactions with miRNA factors in yeast-two-hybrid assays (Figure S2, S5, (Francisco-Mangilet et al. 2015)). Mutation of HYPER RECOMBINATION 1 (HPR1), also known as ERECTA MRNA UNDER-EXPRESSED (EMU) or THO1 (At5G09860), another main component of the THO/TREX complex and homolog of yeast HPR1, also causes a reduction of miRNA levels (Furumizu, Tsukaya, and Komeda 2010). At the same time HPR1 genetically interacts with several major miRNA biogenesis factors: Double mutants of *hpr1* and *hen1, hst*, *se-1* or *ago1-27* show enhancement of the original miRNA-typical single mutant phenotypes (Furumizu, Tsukaya, and Komeda 2010). Analysis of genetic interactions of *tho2* with miRNA biogenesis mutants might thus reveal similar results. Together, the THO/TREX members could potentially influence many different sRNA roads, as their function appears to be rather complementary: While HPR1 affects miRNAs, and THO2 alters si- and miRNAs, TEX1 is required for the production of siRNAs and ta-siRNAs, but seems to be excluded from the miRNA pathway (Yelina et al. 2010). Using a broader selection of mutant alleles for genetic crosses including players of other sRNA pathways could hence help establish a clearer picture of where exactly THO/TREX

components are acting, and where their functions are overlapping in the different pathways. Additional routes of investigation should focus both on the roles of the complex members outside of the sRNA scope, and on the basis of their differential effects on sRNAs. Are these functions exerted as part of the THO/TREX complex, or are complex association and sRNA function uncoupled, and how does the lack of different factors influence interaction with sRNA pathways?

Similar to RCF3 and TGH (Ren et al. 2012; T. Chen, Cui, and Xiong 2015), THO2 interacts with miRNA precursors, indicating that it could be specifically involved in miRNA processing. Consequently, the mutant allele *tho2-5* abolishes recruitment of precursors into the processing complex and reduces association of HYL1 with miRNA precursors (Figure 7b-d, (Francisco-Mangilet et al. 2015)). As mentioned earlier, miRNA processing is suggested to be one of several processes that occur co-transcriptionally. Actors working on miRNA processing thus share 'their' nascent transcript that is produced by RNAPII with a multitude of other proteins that work on capping, or on splicing (reviewed in (Bentley 2014)). Splicing is mediated by the spliceosome, a large complex that contains non-coding RNAs and a profusion of protein factors, and by additional RNA-binding proteins that promote or inhibit splicing, like the Serine Arginine rich (SR) proteins (reviewed in (Meyer, Koester, and Staiger 2015)). Alone due to their proximity, it hence makes sense that there would be crosstalk between proteins of simultaneously active pathways. Some factors simply affect each other or related processes, as does for example the speed of RNAPII the degree of co-transcriptional splicing - the faster RNAPII travels, the smaller the window for splicing (Bentley 2014) -, but others are shared between several processes (Laubinger et al. 2008; Ben Chaabane et al. 2013).

THO2 appears to be a new component in plants shared between the pathways of pri-miRNA processing and splicing. We have connected it with nascent pri-miRNA transcripts, and it is already known from animals and yeast that the THO/TREX complex functions in linking transcription, mRNA splicing and nuclear export (Strässer et al. 2002; Reed and Cheng 2005; Piruat and Aguilera 1998). Our data suggests that also *Arabidopsis* THO2 affects the splicing machinery. While it colocalizes with the canonical spliceosome component SRp34, we did not detect any differences in miRNA factor mRNA or protein accumulation, nor differential splicing patterns of miRNA genes in *tho2* (Figure 7e, S4a-b, (Francisco-Mangilet et al. 2015)). SR34b and other serine/arginine-rich proteins do have

changed splicing patterns in *tho2* and *emu/hpr1* (Figure 7f-g, (Furumizu, Tsukaya, and Komeda 2010; Francisco-Mangilet et al. 2015) - and EMU/HPR1 also colocalizes with the SR protein SR33 (C. Xu, Zhou, and Wen 2015) - indicating that THO2 is, similar to EMU/HPR1, involved in alternative splicing. Interaction with splicing-mediating SR proteins appears to be a common feature of THO/TREX members, and it has even been suggested that it is a conserved part of the mRNA export machinery (Reed and Cheng 2005). Moreover, two serine/arginine-rich splicing factors that associate with the miRNA factor RCF3, namely RS40 and RS41, were recently shown to play roles in the biogenesis of a subset of miRNAs (T. Chen, Cui, and Xiong 2015; Karlsson et al. 2015; T. Chen et al. 2013). Indications for a tight connection between splicing and miRNA biogenesis are thus accumulating, and THO2 could be joining the group of SE, CBC, RCF3 and TGH, all factors with dual roles in miRNA biogenesis and splicing, with SE even displaying similarly lethal null-mutants and *tho2*-like weak mutant phenotypes (Laubinger et al. 2008; Ren et al. 2012; T. Chen et al. 2013). Systematic testing of the effects of all THO/TREX components on differential splicing and sRNA accumulation using RNA and sRNA sequencing approaches, possibly both in mutant and overexpressing lines, would help shed light on the connection between THO/TREX, sRNA and splicing machinery. Due to the sheer complexity of these pathways, genetics alone will however not be able to disentangle the proteins' intertwined functions, and needs to be combined with sophisticated biochemistry approaches.

6.3 Tissue-biased RCF3 and protein modifications as a novel regulatory layer

RCF3 is one of the 26 K-homology (KH) domain encoding genes in *Arabidopsis thaliana*. Since KH domains contain evolutionarily conserved RNA or ssDNA binding motifs (Siomi et al. 1993), and the human KH-type splicing regulatory protein KSRP is associated both with mRNA decay and DROSHA and DICER complexes (Gherzi et al. 2006; Ruggiero et al. 2007; Trabucchi et al. 2009), RCF3 was a promising miRNA cofactor candidate. Confirming this notion, several recent publications established a role for RCF3 in the context of transcriptional modification processes and splicing as well as in miRNA processing (Jeong et al. 2013; T. Chen et al. 2013; Jiang et al. 2013; T. Chen, Cui, and Xiong 2015).

Although the spectrum of RCF3 activity thus is rather broad, expression of the gene distinctively peaks in young dividing tissues, i.e. the vegetative and reproductive apex (Figure 2, (Karlsson et al. 2015)). There, we see the clearest effects of *RCF3* mutation, which causes a strong decrease of mature miRNA levels and a subsequent increase of mRNA target accumulation (Figure 3-4, (Karlsson et al. 2015)). We thus propose that actual RCF3 function is strongly biased to these tissues (Karlsson et al. 2015). This also illustrates one of the great advantages of having a model system like *Arabidopsis* as opposed to working with cell cultures, since this type of factor can only be discovered in the context of a complex organism.

Similar bias of a miRNA biogenesis pathway component has been described previously, and also expression or activity of miRNAs themselves for instance oftentimes is specific to a certain tissue type or a developmental stage. One plant example for this is the phase-change involved miR172. It accumulates in flower primordia upon floral induction, while at the same time levels of the counteracting miR156 are decreasing (Wollmann et al. 2010; G. Wu and Poethig 2006; J.-W. Wang, Czech, and Weigel 2009). Other miRNAs, but also miRNA biogenesis factors, can be influenced by environmental contexts, like biotic or abiotic stress conditions (for example (Khraiwesh, Zhu, and Zhu 2012; Fujii et al. 2005)). A feature often observed is hyper- or hyposensitivity of miRNA biogenesis mutants to the stress-related plant hormone abscisic acid (K. Earley et al. 2010; Hugouvieux, Kwak, and Schroeder 2001). Development- and tissue-biased activity of miRNA factors has also been widely recognized in animals. One thoroughly studied protein in this context is the highly conserved RNA-binding LIN28. It is specifically expressed during early developmental stages, in undifferentiated tissues and poorly differentiated tumors, and decreases with developmental progression and age. Via binding to precursor transcripts of the differentiation-promoting *let-7* RNA, LIN28 inhibits *let-7* processing, thus helping to maintain pluripotency (reviewed in (Tsialikas and Romer-Seibert 2015)). In plants, there has been little published evidence for biased activity of a miRNA factor. Identification of *RCF3* thus establishes tissue-biased expression (and activity) as a novel layer of fine-tuning and regulation also within the plant miRNA biogenesis pathway.

For now, most factors and many miRNAs are thought to be expressed ubiquitously. In-detail analysis, as described here for *RCF3*, can however reveal distinct

variations of expression patterns. Exemplary for this are *miR172* and its target mRNA *AP2*. Both had previously been reported to be broadly expressed. A critical advance in our understanding of their modes of action in the context of flower development came from the observation that they have quite exquisite differential expression patterns, depending on phase transition and specific tissues (Wollmann et al. 2010). One way to find additional biogenesis factors that are similarly differentially regulated (and regulating) would be to use tissue-specific RNA-pull down, extracting miRNAs and miRNA associated RNAs with distinct expression or action patterns and analyzing associated proteins (Slobodin and Gerst 2011; Iioka et al. 2011). Moreover, cofactor screens such as our luciferase-based approach can be employed to look for organ-specific luciferase reactivation caused by this kind of specialized biogenesis cofactors. Combining these types of methods to scout the research avenue of tissue-biased factors is going to further advance our knowledge on how general pathways can have tissue-specificity, as it is for example seen in alternative splicing (Schindler et al. 2008).

Known candidates for tissue-biased regulation in plants are the shoot apical meristem biased DRB2, a close relative of DRB1/HYL1 (Eamens et al. 2012; Reis et al. 2015), and the protein kinase MPK3. The latter is a potential antagonist of the phosphatases CPL1/2 in regulating phosphorylation-status (and thus activity) of RCF3 and HYL1 (Raghuram et al. 2015; Karlsson et al. 2015). Both RCF3 and HYL1 are thought to be recruited to the pri-miRNA processing complex in their active, hypophosphorylated isoforms (Manavella et al. 2012; Raghuram et al. 2015; T. Chen, Cui, and Xiong 2015). Though the specific RCF3 mode of action is still unclear, we speculate that it might be a stimulator of CPL1/2 phosphatase activity. Inversely correlated expression of the phosphatase-antagonist MPK3 in comparison with RCF3 - its transcript levels are low in apices - could then explain the notably higher HYL1 activity and consequently elevated miRNA biogenesis observed in this tissue niche (Figure S8, (Karlsson et al. 2015; Schmid et al. 2005)).

With the identification of a shoot apex-biased role in changing the phosphorylation status of HYL1 (Figure 6, (Karlsson et al. 2015)), our analysis of RCF3 combines hence two themes that are very likely among the predominating features of cofactors yet to be discovered in the plant miRNA context: tissue-biased or -specific action, and modification of already known factors within miRNA biogenesis. Under

certain circumstances, such as active cell division, a general regulation of conserved factors such as HYL1 can be expected, since a large number of different miRNAs are involved in cell cycle coordination and can thus be steered collectively. More specific situations, for example cell type specific regulation, would on the other hand profit more from the precise regulation of single miRNAs and their targets, leaving other miRNAs and targets unaffected.

Different types of protein modifications have already been described to be important in various steps of animal miRNA biogenesis. Acetylation, for example, stabilizes DROSHA, a microprocessor complex protein without homologs in plants, through inhibition of ubiquitin-mediated degradation (X. Tang et al. 2013). Phosphorylation of DROSHA on the other hand is required for its nuclear localization (X. Tang et al. 2010). Similarly, the localization of human AGO2 in P-bodies depends on its phosphorylation, as does downregulation of AGO2-mediated mRNA target cleavage activity and subsequent increase of translational repression. Adding a further layer of specificity to these processes, both AGO2-phosphorylations are mediated by two distinct kinases (Horman et al. 2013; Zeng et al. 2008). Similar mechanisms have been indicated in plants, since for example dephosphorylation of RCF3 appears to be required for RCF3 subcellular localization (T. Chen et al. 2013; T. Chen, Cui, and Xiong 2015). Biochemical analysis of main miRNA biogenesis players like AGO1 or SE, and further exploration of the changes, circumstances and consequences of HYL1 and DCL1 phosphorylation status will thus reveal more details of this still largely unexplored regulatory layer of miRNA biogenesis (Engelsberger and Schulze 2012). In addition, subcellular localization is another feature that to date has not been explored much in plants and might hence be a worthwhile area of investigation.

6.4 *HWS* and sensitized backgrounds for specialized factor-fishing

Mutation of the candidate gene *HWS* causes a clear suppression of the MIM156 phenotype (Figure 1a, manuscript in preparation). The effect of *hws* - besides rescuing phenotypic abnormalities of MIM156 plants it also suppresses the phenotypes of MIM164, MIM159 and MIM319 - is conspicuous in the transgenic MIM system, and the characteristic ‘skirt’ phenotype based on the defect in sepal separation is easy to spot

(Figure 2a, d, manuscript in preparation). Yet, determination of HWS' molecular role and mode of action within miRNA biogenesis in the undisturbed natural system is not straightforward.

Analysis of steady-state levels of miRNAs and their targets in the *hws* and *35S:HWS* lines reveals weak effects fitting the profile of a negative biogenesis regulator (Figure 3b-d, manuscript in preparation). Additional arguments support a connection of HWS with miRNA biogenesis: Overexpression of HWS causes phenotypes that strongly resemble mutants like *hyl1-2* or *ago1-27*, and double mutants of *hws* with a set of major miRNA biogenesis factors display clear epistatic interactions (Figure 3a, manuscript in preparation).

In this context, the HWS F-box domain provides a first hint at a potential HWS-mode of action. The domain is strongly connected with HWS functionality, as shown by failed skirt complementation and lack of overexpressor phenotype upon transformation of *hws-1* with *35S::mHWS* that lacks the F-box (Figure 4b, 5a, S1a-c, manuscript in preparation). F-box proteins impart specificity to SCF-complexes, to control which substrates are targeted by these E3 ligases for ubiquitination and subsequent degradation (Risseeuw et al. 2003). Our mass spectrometry analysis indicates that HWS as well is part of such a complex, as full-length HWS, but neither mHWS nor a GFP control associated with the SCF-complex compounds SKP1, SKP2 and CUL1 (manuscript in preparation).

Involvement of F-box mediated processes in miRNA function is not without precedent. AGO1 is targeted and destabilized by both the *Arabidopsis* FBW2 and the virus-derived silencing suppressor P0 (K. Earley et al. 2010; Bortolamiol et al. 2007; Nicolas Baumberger et al. 2007). Even for animal AGO-proteins like AGO2, ubiquitination and coupled proteasomal degradation is a typical way of activity modification (reviewed in (Jee and Lai 2014)). Ubiquitination is hence another aspect of the protein-modification guided regulatory layer of miRNA biogenesis. Attachment of different numbers of ubiquitin molecules to a protein further provides means of influencing miRNA factors not only via degradation, but also affecting for example associated cellular signaling or intracellular trafficking (Mukhopadhyay and Riezman 2007; W. Li and Ye 2008).

However, interaction between HWS and its targets could be transient, and thus hard to observe experimentally. In the MS assay, AGO1 was the sole most relevant

miRNA-related protein that was at least close to significantly enriched. Transient co-expression of HWS with and without its F-box with AGO1 nonetheless did not result in clear changes of AGO1 accumulation, or of its ubiquitination status (Figure S5b, manuscript in preparation). Thus, how exactly HWS exerts its effects, and which factors are affected by HWS-mediated regulation remains unclear. Large-scale analysis of miRNA factor ubiquitination using existing datasets (Walton et al. 2016), coupled with experimental verification of specific proteins' ubiquitination levels in wild-type and mutant plants (L. Liu et al. 2010) could be one way to investigate not only the role of HWS within ubiquitination and miRNA biogenesis, but also the role of ubiquitination itself in miRNA biogenesis in general.

Furthermore, as a direct effect of HWS on the protein level, on the abundance of a potential target, or on ubiquitination, could not be detected, an obvious follow-up experiment is the exploration of indirect HWS effects, looking at transcript levels using RNA-seq. With samples both with and without the MIM156-context, it will be possible to dissect the contributions of HWS within the MIM-pathway as well as its broader role in general miRNA function.

Clearly, the use of sensitized backgrounds to screen for more specialized cofactors of miRNA biogenesis serves its purpose, as it enabled us to identify HWS and several other, to date uncharacterized factors. As expected, the effect of *hws* is most obvious within the artificial MIM background, and much less pronounced under more natural circumstances. This poses the question of how much the choice of a sensitized background is indeed beneficial in the end. It certainly helps to find more factors - but deciphering their biological role in a natural context can be challenging.

6.5 How to find missing pieces and integrate everything into the big picture?

We know that gene expression is an important regulator of cell differentiation, specification and patterning. A major factor contributing to differential expression are sRNAs, and, more specifically, also miRNAs. Since the broad framework of miRNA biogenesis and action is now set, it is time to connect the pathway factors and cofactors that we know, with the surrounding landscape of biological processes. In this thesis, I characterized RCF3, THO2 and HWS in the broad miRNA context, adding information

both to different layers of the miRNA regulatory framework and to its extended background.

MiRNA factors and cofactors known so far mostly act in a rather general fashion. Discovery of the strongly tissue-biased cofactor RCF3 draws attention to a new level of complexity in miRNA biogenesis and function. It serves as reminder that, instead of looking at a whole organism, we also need to zoom in on specific organs, tissues, and even single cells, or explicitly defined developmental windows, both for the detection of additional factors and to see which factors contribute how to expression variation. Substantiation of the co-transcriptionality of pri-miRNA processing in the context of THO2 on the other hand puts the idea forward that, besides zooming in, we also have to broaden the scope and not look at the miRNA pathway as an island within the organism, but rather one of many simultaneous processes like splicing, that are connected on several levels. Taking this into account when trying to fit in new 'miRNA cofactors' will help to explain their actual roles - in the miRNA context as well as elsewhere. Finally, the potential connection of HWS with ubiquitination as well as miRNA factor phosphorylation in the RCF3 context confirms protein modifications as an additional regulatory layer in the miRNA pathway. With this novel layer in mind, we should revisit already established factors and cofactors to for example see nuances in activity related to suchlike modifications that we missed before. Potentially, this can not only reveal further details of known, complex regulatory steps, but also connect miRNAs to other existing mechanisms such as phosphorylation, and thereby add novel factors to the picture.

With this, new challenges for miRNA biology research have been established: zooming in on specific sections, while at the same time widening the scope to related pathways, as well as incorporating the protein-modification regulatory layer. For integration of those challenges, together with further newly found factors, into the large miRNA puzzle, the work here shows that genetics is a powerful approach, but can rapidly reach its limits when contexts are too intertwined, or based on subtle biochemical changes. Then, it is necessary to become more interdisciplinary, and combine traditional genetics research with biochemistry approaches. In this way, already known, but not yet satisfactorily characterized cofactors as well as newly found candidates can be placed in the right position.

Apart from actual, physical experiments, the ever progressing high-throughput methods of the sequencing era present innumerable opportunities for the *in silico* supported quest for cofactors and their roles. To this end, sequencing-based datasets from RNA-seq, GWA or methylation studies can be combined with interactome networks, deduced either from experimental data or predicted, based on analyses in other systems (for example (Arabidopsis Interactome Mapping Consortium 2011; Y. Wang et al. 2014)), to unravel some of the remaining mysteries, as has for example been done in a recent network-based analysis of schizophrenia GWAs (I. Lee et al. 2010; Chang et al. 2015). A large-scale project like the Encyclopedia of DNA Elements (ENCODE) could in this context be an additional source of valuable information (Lane et al. 2014). Coupling obtained *in silico* data with molecular wet lab methods, computationally identified candidates can be validated and characterized the traditional way. Future projects should thus both exploit existing datasets, but also take advantage of state-of-the-art methods to generate tailored information, for example using single cell RNA- or methylome sequencing (F. Tang et al. 2009).

Lastly, while it is definitely worthwhile to spend more efforts on clarifying and extending the so-far known miRNA picture, it would be beneficial to not only focus on the components and mechanics of the pathway, but to rather look at it in the context of a broader evolutionary perspective (Ma, Coruh, and Axtell 2010; Vazquez et al. 2008; Smith et al. 2015). The hypothesis that it is regulators within a gene's expression network and not major genes themselves that get mutated for adaptation in the course of evolution (deduced from (López-Maury, Marguerat, and Bähler 2008)) places miRNAs in the spotlight as important modulators of gene expression. Extracting how miRNAs and their regulators evolve over time, both within and between species, would advance our understanding of their contribution to adaptation and evolution, and could tell us where regulatory steps originated in the first place. A valuable source of information in this context is the natural variation of the miRNA machinery and its fine-tuning. Studies in different species can reveal striking similarities, such as for miR156 and its evolutionarily conserved role in vegetative phase change in annual herbaceous plants and perennial trees (J.-W. Wang et al. 2011), but also differences, as seen for the maize *dcl1* mutant *fuzzy tazzle* (Thompson et al. 2014). Both similarities, and differences, that we observe in miRNAs and cofactors of various accessions or species, and their genetic as well as

environmental backgrounds can enable us to infer these proteins' roles in and impact on miRNA biogenesis in particular and plant regulatory systems within an evolutionary context in general.

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To Petter, you are the root.

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Publications

Karlsson P et al. (2015), KH domain protein RCF3 is a tissue-biased regulator of the plant miRNA biogenesis cofactor HYL1, PNAS 11(45): 14096-14101

Francisco-Mangilet AG*****, **Karlsson P*** et al. (2015), THO2, a core member of the THO/TREX complex, is required for microRNA production in Arabidopsis, Plant J 82: 1018-1029

Pietra S*****, **Lang P*** & Grebe M (2014), *SABRE* is required for stabilization of root hair patterning in *Arabidopsis thaliana*, Physiologia Plantarum 153:440-453

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Tübingen, October 2016
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THO2, a core member of the THO/TREX complex, is required for microRNA production in Arabidopsis

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SUMMARY

The THO/TREX complex mediates transport of nascent mRNAs from the nucleus towards the cytoplasm in animals, and has a role in small interfering RNA-dependent processes in plants. Here we describe five mutant alleles of *Arabidopsis thaliana THO2*, which encodes a core subunit of the plant THO/TREX complex. *tho2* mutants present strong developmental defects resembling those in plants compromised in microRNA (miRNA) activity. In agreement, not only were the levels of siRNAs reduced in *tho2* mutants, but also those of mature miRNAs. As a consequence, a feedback mechanism is triggered, increasing the amount of miRNA precursors, and finally causing accumulation of miRNA-targeted mRNAs. Yeast two-hybrid experiments and confocal microscopy showed that THO2 does not appear to interact with any of the known miRNA biogenesis components, but rather with the splicing machinery, implying an indirect role of THO2 in small RNA biogenesis. Using an RNA immunoprecipitation approach, we found that THO2 interacts with miRNA precursors, and that *tho2* mutants fail to recruit such precursors into the miRNA-processing complex, explaining the reduction in miRNA production in this mutant background. We also detected alterations in the splicing pattern of genes encoding serine/arginine-rich proteins in *tho2* mutants, supporting a previously unappreciated role of the THO/TREX complex in alternative splicing.

Keywords: *Arabidopsis thaliana*, gene silencing, micro RNA, small RNAs, THO2, THO/TREX complex.

INTRODUCTION

Among the mechanisms for post-transcriptional gene silencing in plants, small RNA-dependent gene regulation plays a central role (Baulcombe, 2004). MicroRNAs (miRNAs) are a specific class of small RNAs (mostly 21–22 nucleotides long) that mediate endogenous gene silencing (Jones-Rhoades *et al.*, 2006). In plants, DICER-LIKE1 (DCL1) processes mature miRNAs from long primary miRNA transcripts (pri-miRNAs) that form a stem–loop secondary structure. During processing in nuclear dicing bodies, DCL1 requires the assistance of the zinc finger protein SERRATE (SE) and the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1) for accurate excision of the miRNAs (Fang and Spector, 2007; Fujioka *et al.*, 2007; Voinnet, 2009). The mature miRNAs associate with an ARGONAUTE (AGO) protein and guide the RNA-Induced Silencing Complex (RISC) Complex, through sequence complementarity, to their target mRNAs, ultimately silencing them. In contrast, production of small interfering RNAs (siRNAs), a different class of small RNAs, is based on processing of a highly complementary double-stranded RNA by a DCL1-independent pathway. In a very specialized pathway, *trans*-acting siRNAs (tasiRNAs) and secondary siRNAs are produced from *Trans-acting siRNA (TAS)* or even mRNA transcripts after initial cleavage by a miRNA (Chapman and Carrington, 2007). The production of tasiRNAs follows a particular process that shares components with both the miRNA and siRNA biogenesis pathways.

In yeast and animals, the THO/TREX complex has been characterized as a multimeric protein complex that mediates transcription elongation (yeast), splicing of mRNAs (animals), and export of mRNAs from the nucleus (both yeast and animals) (Reed and Cheng, 2005). In all studied organisms, the complex comprises TEX1 and several THO subunits as well as accessory proteins (Dufu *et al.*, 2010; Moon *et al.*, 2011; Gewartowski *et al.*, 2012). A plant THO core complex, similar to the metazoan THO/TREX complex, has been identified in Arabidopsis. It consists of at least eight proteins: THO1/HPR1/EMU, THO2, THO3/TEX1, THO4, THO5, THO6, THO7 and UAP56 (Furumizu *et al.*, 2010; Jauvion *et al.*, 2010; Yelina *et al.*, 2010). Mutations in the Arabidopsis *TEX1*, *THO6* and *THO1* genes cause a reduction in the siRNA levels from *TAS* genes, inverted repeat (IR) genes and transgenes (Jauvion *et al.*, 2010). The *tho1* mutant alleles were also found to affect the alternative splicing patterns of transcripts encoding serine/arginine-rich proteins (Furumizu *et al.*, 2010).

To date, the functional specialization of other plant THO components in plants has not been fully dissected. Among them is the core component THO2, which we investigated here. The yeast gene encoding THO2 was first identified as a gene affecting transcription elongation of HYPERRECPMBINATION PROTEIN 1 (HPR1) (Piruat and Aguilera, 1998). In Arabidopsis, null mutations in *THO2* cause embryo lethality, whereas null mutations in *THO1* and *TEX1* (*hpr1* and *tex1*, respectively) cause developmental defects including dwarf stature, leaf serration, curly leaves and embryonic defects (Furumizu *et al.*, 2010; Jauvion *et al.*, 2010; Yelina *et al.*, 2010). The embryo lethality of *tho2* mutants suggests that THO2 has essential roles that go beyond those of other members of the complex. However, that lethality is a major problem with respect to the study of THO2 functions, and has hampered our understanding of the functional specialization of THO2.

Here we elucidated the function of Arabidopsis *THO2* by exploring a set of five mutant alleles ranging from null to hypomorphic. The identification of viable *tho2* mutant alleles allowed us to study the functions of THO2. We showed that plants containing mutations in the *THO2* gene present serious developmental defects, with failures in all the small RNA pathways that we examined, including miRNAs, tasiRNAs and siRNAs. As a consequence of the reduced production of miRNA, a feedback mechanism is triggered, increasing the amount of miRNA precursors and causing a concomitant over-accumulation of miRNA-targeted mRNAs. We found that THO2 does not appear to interact with any of the known miRNA biogenesis components, but rather with the splicing machinery, implying an indirect role of THO2 in small RNA biogenesis. Our studies showed that THO2 interacts with miRNA precursors, assisting their transport into the miRNA-processing complex. In *tho2* mutant plants, the miRNA precursors fail to associate with the processing complex, specifically with HYL1, explaining the reduction in miRNA production observed in this mutant background. We also found that alternative splicing was compromised in *tho2* mutants, possibly reflecting a second conserved role for THO2. The severity and multiplicity of the molecular pathways affected in *tho2* mutants may explain why mutations in this gene cause more severe developmental problems than the lack of any other component of the THO/TREX complex.

RESULTS

Identification and characterization of several Arabidopsis *THO2* mutants

The study of the biological functions of THO2 was particularly challenging until now because no homozygous *tho2* alleles had yet been isolated. Previously studied T-DNA mutant lines for *THO2*, specifically the *tho2-1* (SALK_072011c) and *tho2-2* (SALK_130342) mutants, aborted at embryonic stages (Furumizu *et al.*, 2010; Jauvion *et al.*, 2010; Yelina *et al.*, 2010). These mutants feature T-DNA insertions in exons 16 and 18, respectively (Figure 1a). Consistent with these previous reports, we failed to identify mature plants homozygous for these alleles. However, we observed a population of tiny seedlings, which accounted for approximately 6% of the *tho2-1* and *tho2-2* segregation populations, after seeds were sown on MS plates. These seedlings never developed true leaves, had very short roots, failed to develop and died (Figure 1b,c). PCR analysis confirmed that all these tiny seedlings were homozygous for T-DNA insertions at the *THO2* locus (Figure 1d). These results showed that even though most *tho2-1* and *tho2-2* homozygous embryos abort, some reached very early developmental stages and may be used for further experimentation.

Recently a genetic screen using an artificial microRNA (amiRNA) targeting the luciferase reporter gene was performed to identify proteins acting in the miRNA pathway (Manavella *et al.*, 2012a). Whole-genome sequencing followed by SHORE mapping allowed mapping of the locus responsible for the miRNA dysfunction in one of the isolated mutant plants to chromosome 1, where a new polymorphism in the *THO2* (*At1g24706*) gene was identified. This mutant, *tho2-5*, contains a C→T mutation that results in a non-synonymous substitution of serine by phenylalanine (Figure 1a). The *tho2-5* plants showed a dwarf stature and narrow curly leaves, and produced few seeds (Figure 2a–c). Transformation of the mutants with the wild-type *THO2* cDNA, driven by its own promoter and fused to mCitrine, restored silencing of the luciferase reporter and reversed the *tho2-5* morphological defects, confirming that the mutation in *THO2* is the cause of the observed phenotype (Figure 2a–c). A second mutant, *tho2-6*, was isolated from an activation tagging screening performed in our laboratory. The position of the T-DNA insertion was estab-

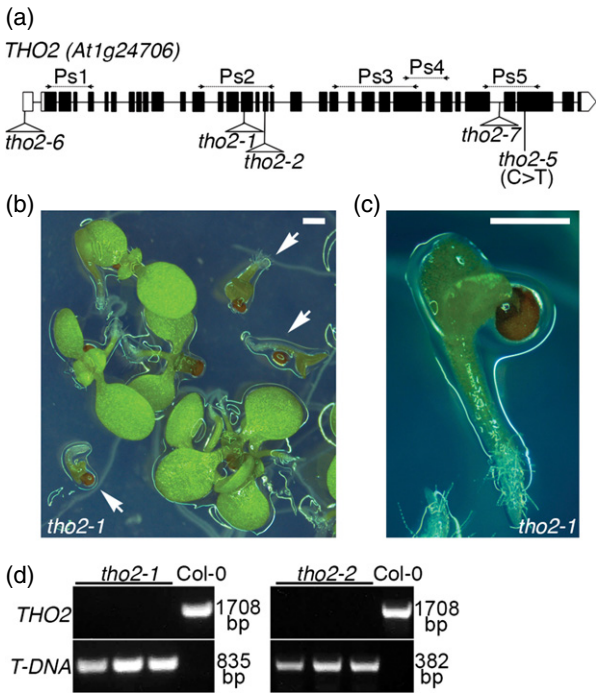

Figure 1. Identification of *tho2-1a* and *tho2-2* homozygous plants.
(a) Gene structure of *THO2* showing single nucleotide substitution and T-DNA insertion sites in *tho2-1*, *tho2-2*, *tho2-5*, *tho2-6* and *tho2-7*. Black boxes and lines represent exons and introns, respectively; white boxes represent 5′ and 3′ UTRs. Arrows indicate the position of primer sets used for RT-PCR analysis in Figure 5(a).
(b) Fourteen-day-old seedlings of *tho2-1* grown on an MS plate, showing arrested growth. Arrows indicate *tho2* homozygous seedlings. Scale bar = 1 mm.
(c) Magnified view of homozygous seedling shown in (b). Scale bar = 1 mm.
(d) PCR genotyping of homozygous *tho2-1* and *tho2-2* seedlings.

lished by TAIL-PCR, and mapped to the 5′ UTR of *THO2* (Figure 1a). Segregation analysis and PCR-assisted genotyping allowed us to isolate homozygous *tho2-6* plants that contain a single copy of the T-DNA insertion. These mutant plants developed curly leaves and had a bushy reduced stature (Figure 3a–c). The plants present an abnormal distribution of petals (Figure 3d), atrophic anthers that fail to produce mature pollen grains leading to complete sterility (Figure 3e), fusion of the carpels that form twisted pistils exposing the ovules (Figure 3f), and a large number of trichomes in the floral buds (Figure 3g). Inspection of dissected siliques from heterozygous plants revealed that 15% of the ovules (n = 553), which are probably homozygous for the T-DNA insertion, aborted (Figure 3h,i).

Additionally, we analyzed an uncharacterized *tho2* allele (SALK_144229) that contains a T-DNA insertion in intron 30 towards the end of the gene (Figure 1a). Plants homozygous for this allele, named *tho2-7*, were able to reach maturity, showing small stature, serrated leaves, an increased number of trichomes on their sepals, and short anther filaments and pistils (Figure 4a–f). Approximately 5% of the flowers of mutant plants had five petals (Figure 4d). Their fertility was reduced to less than 20% compared to heterozygous plants due to embryonic abortion (Figure 4h,i).

Expression of *THO2* in wild-type and mutant Arabidopsis plants

RT-PCR expression analysis of *THO2*, using primers sets designed to flank the mutation sites (Figure 1a), revealed that *tho2-1*, *tho2-2* and *tho2-7* produced truncated versions of the *THO2* mRNA (Figure 5a). *tho2-6* showed intact but strongly reduced levels of *THO2* mRNA, while *tho2-5*, as expected from plants with a non- synonymous point mutation, showed no change in the abundance or size of the mRNA (Figure 5a). Considering the mutant phenotypes, it is conceivable that *tho2-1* and *tho2-2* are null alleles, *tho2-6* is a knockdown allele, and *tho2-5* and *tho2-7* are hypomorphic. Thus the new mutant alleles *tho2-5*, *tho2-6* and *tho2-7* may become very useful tools to study the biological function of THO2, the core protein of the THO/TREX complex. RT-PCR experiments showed that, in wild-type plants, *THO2* is expressed at very similar levels in the flower buds, mature flowers, stems, leaves, roots and whole seedlings, as well as in pollen at all developmental stages, including isolated spores, unicellular, bicellular and tricellular phases, and mature pollen (Figure 5b).

THO2 is required for accumulation of miRNAs, tasiRNAs and siRNAs

Components of the Arabidopsis THO/TREX complex, such as TEX1 and THO1, have been described as important part-

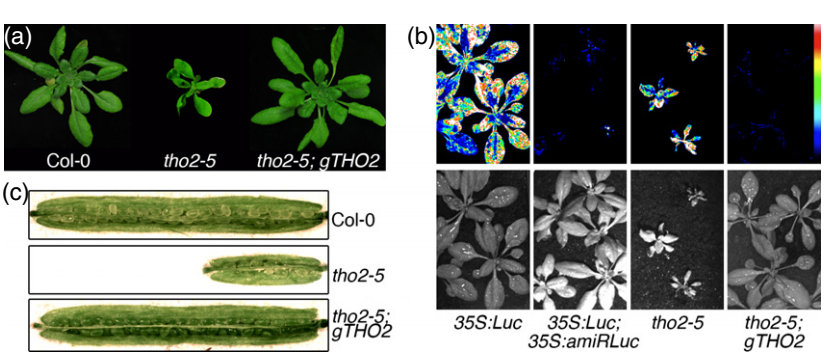

Figure 2. Isolation and characterization of *tho2-5*.
(a) Phenotype of 20-day-old Col-0, *tho2-5* mutant and complemented plants.
(b) Bioluminescence phenotype of *tho2-5* mutants, complemented mutants, reporter lines (35S:Luc;35S:amiRLuc) and Pro35S:LUC controls. The upper panels show bioluminescence activity; the colored scale indicates low luminescence (blue) to high luminescence (white). The lower panels show bright-field images of the same plants.
(c) Dissected siliques of Col-0, *tho2-5* and the complemented mutant.

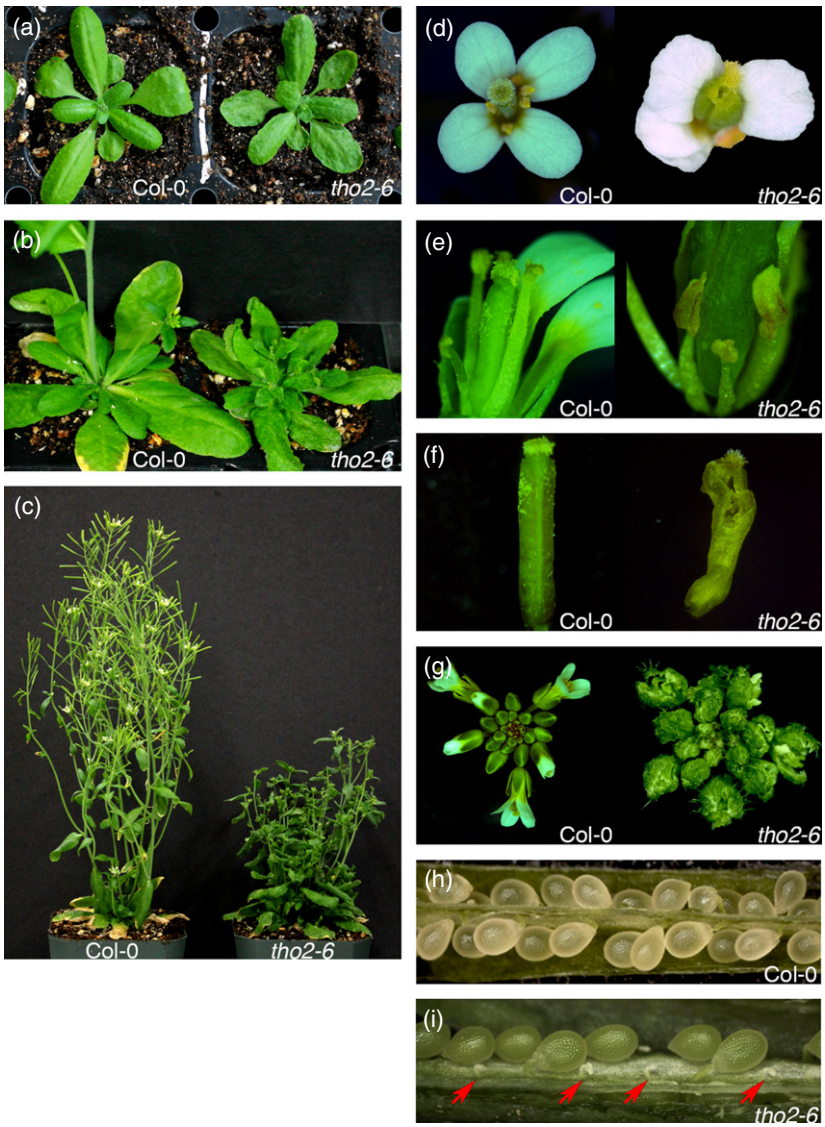

Figure 3. Phenotypic characterization of *tho2-6* mutant plants.
(a) *tho2-6* and Col-0 plants at the early rosette-leaf stage.
(b) Leaf proliferation and delayed bolting in *tho2-6* plants.
(c) *tho2-6* and wild-type plants at 42 days old.
(d–g) Flowers, anthers, pistils and inflorescences of wild-type and *tho2-6* plants.
(h, i) Dissected siliques of wild-type and *tho2-6* plants. Red arrows indicate aborted ovules.

ners in several small RNA pathways (Furumizu *et al.*, 2010; Jauvion *et al.*, 2010; Yelina *et al.*, 2010). To test whether THO2 is involved in siRNA biosynthesis, we crossed *tho2* mutants (*tho2-6* and *tho2-7*) with *JAP3* transgenic lines. These transgenic plants express an inverted repeat version of the PHYTOENE DESATURASE (*PDS*) gene under the control of the phloem-specific *SUC2* promoter (SUC2p: PDS-IR; Smith *et al.*, 2007). *JAP3* plants exhibit a unique phenotype of photo-bleaching around the leaf veins, consequence of the silencing of the *PDS* gene by the SUC2p: PDS-IR construct. Segregation analysis and PCR-assisted genotyping revealed that wild-type plants or plants heterozygous for the *tho2-6* and *tho2-7* alleles retain the typical *JAP3* photobleaching phenotype (Figure 6a). In contrast, all plants homozygous for the mutations completely lost the photobleaching phenotype and showed the typical *tho2-6* or *tho2-7* leaf shape (Figure 6a). Quantitative RT-PCR analyses demonstrated that the level of *PDS* transcripts was higher in the *JAP3/tho2-6* and *JAP3/tho2-7* plants than in the original *JAP3* line (Figure 6b). This over-accumulation of the *PDS* transcript correlated with a reduction in *PDS*-derived siRNAs observed in both the *tho2-6* and *tho2-7* mutant backgrounds (Figure 6c).

As the *tho2-5* allele was isolated from a miRNA-activity based screening, we wished to determine whether THO2 also has a role in the miRNA pathway. To determine whether *THO2* is required for miRNA biogenesis or action, we evaluated the steady-state levels of mature miRNAs and miRNA-targeted mRNAs in *tho2* mutants by Northern blotting and quantitative RT-PCR, respectively. We found that miRNA levels were strongly reduced in all tested *tho2* mutants, with the exception of *tho2-7*, which showed normal miRNA levels (Figure 6d). Interestingly, no changes were detected for miR171, which suggests that THO2 is not

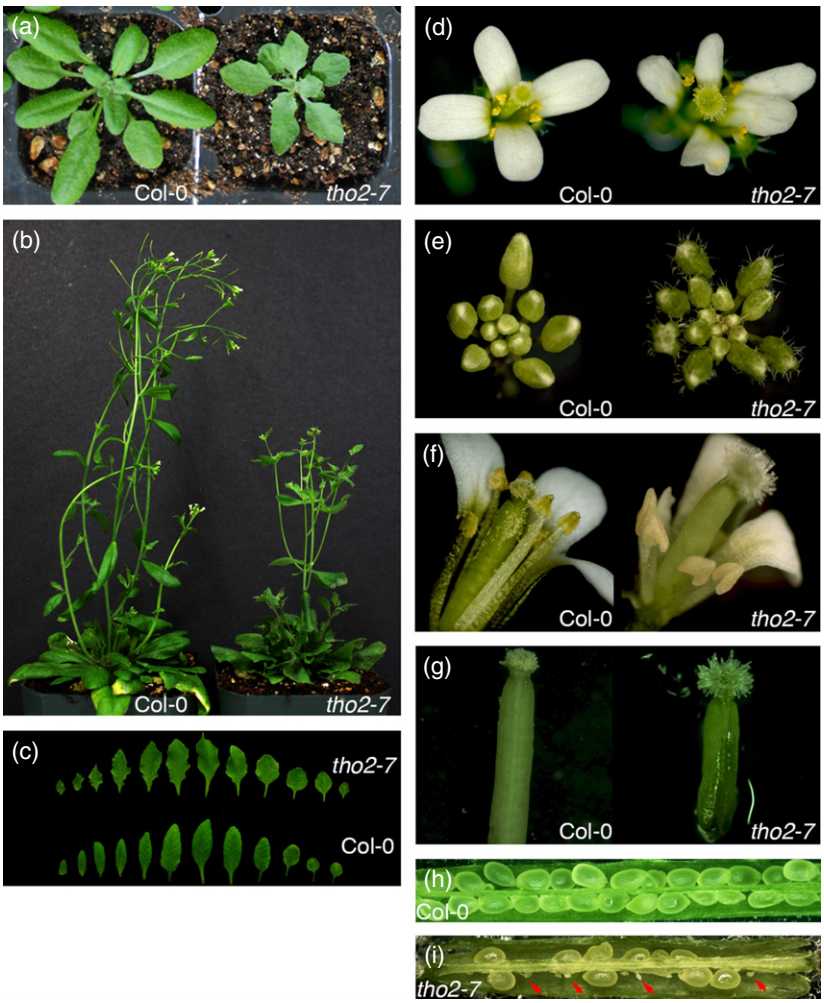

Figure 4. Phenotypic characterization of *tho2-7* mutant plants.
(a) *tho2-7* and Col-0 plants at the early rosette-leaf stage.
(b) *tho2-7* and Col-0 wild-type plants at 42 days old.
(c) Leaf series of *tho2-7* showing serration of leaf edges compared with wild-type leaves.
(d–g) Flowers, inflorescences, anthers and pistils of wild-type and *tho2-7* plants.
(h, i) Dissected siliques of wild-type and *tho2-6* plants. Red arrows indicate aborted ovules.

involved in generation of this miRNA. The reduction in miRNA levels was paralleled by higher accumulation of several miRNA-target mRNAs (TCP3 targeted by miR319; MYB33 targeted by miR159; ARF8 targeted by miR167; AP2 and TOE2 targeted by miR172) (Figure 6e). All tested *tho2* mutant alleles showed higher levels of miRNA precursors than wild-type plants (Figure 6f). Such accumulation of miRNA precursors is a common transcriptional feedback response to low mature miRNA levels, as observed in plants mutated in other miRNA biogenesis factors (Song *et al.*, 2007; Laubinger *et al.*, 2008; Ben Chaabane *et al.*, 2013; Wu *et al.*, 2013). The primers used to detect miRNA precursors in this work cannot distinguish between pri-miRNA and pre-miRNA sequences. We have used the term miRNA precursors as a general term to refer to both RNA intermediate molecules. As shown for mutants of other THO/TREX complex components (Jauvion *et al.*, 2010; Yelina *et al.*, 2010), all tested *tho2* mutants showed a marked reduction in secondary siRNA derived from *TAS1* and endogenous siRNA (Figure 6d). We observed that inclusion of a *THO2* genomic fragment in the *tho2-5* mutant background reverses the reduction in small RNAs, thus confirming that THO2 is responsible for this phenotype (Figure S1a). For both the RNA blots (Figure 6d) and the quantitative RT-PCR experiments (Figure 6e,f) we used *hyl1-2* mutants as control plants. Mutants in *hyl1* show reduced production of miRNAs, over-accumulation of miRNA precursors, and a concomitant increase in miRNA-targeted transcripts (Han *et al.*, 2004; Song *et al.*, 2007).

THO2 is associated with miRNA precursors and is required for their recruitment into the miRNA-processing complex

With the aim of understanding how THO2 acts in the miRNA pathway, we co-transformed plants with THO2:eGFP and HYL1:mCherry constructs, and observed the localization of the proteins using a confocal microscope. Our observations revealed that THO2 accumulated in the nucleus, specifically in nuclear speckles different to those in which the miRNA biogenesis machinery is located (Figure 7a). The same nuclear expression pattern was observed for *tho2-5* mutants rescued using the gTHO2:mCitrine construct (Figure S1b). In accordance with this

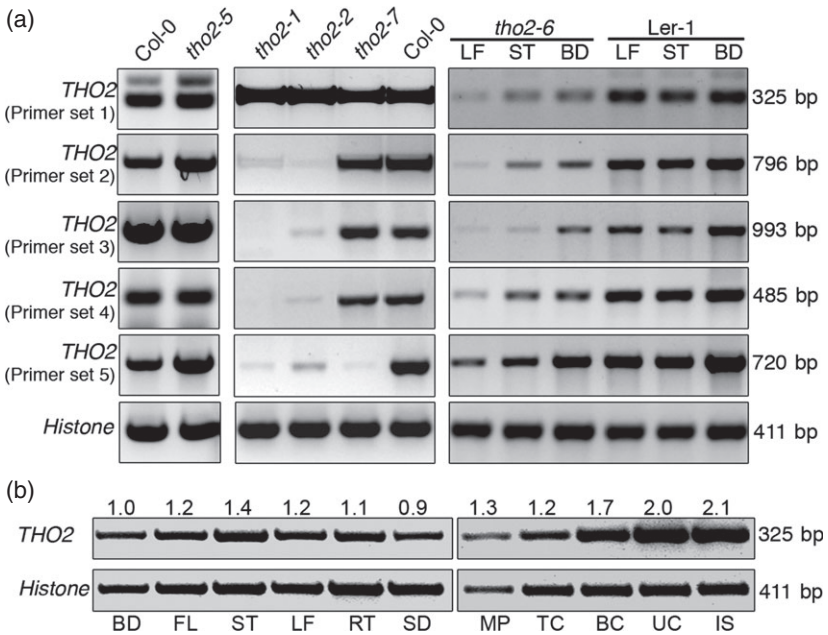

Figure 5. Expression of *THO2*.
(a) Expression of *THO2* measured by RT-PCR in *tho2-1*, *tho2-2*, *tho2-5*, *tho2-6* and *tho2-7* plants. For *tho2-6*, tissues from leaf (LF), stem (ST) and floral buds (BD) were analyzed. Histone was used as a loading control. The positions of primer sets used for the analysis are shown in Figure 1(a).
(b) RT-PCR analysis of THO2 expression in Col-0 floral bud (BD), open flower (FL), stem (ST), leaf (LF), root (RT), and whole seedling (SD), and at different developmental stages of Col-0 pollen: mature pollen (MP), pollen in tricellular (TC), bicellular (BC), unicellular (UC) phases and isolated spores (IS). Histone was used as control.

observation, we were not able to detect any interaction in a yeast two-hybrid interaction screen using THO2 fused to GAL4-BD and a collection of 18 known small RNA-related proteins fused to GAL4-AD (Figure S2).

The facts that THO2 does not interact with any miRNA biogenesis factor and that it is also involved in other small RNA pathways suggests that it may act in early steps that are common to all these pathways, such as transport or stabilization of the primary double-stranded RNA precursors. In order to test whether THO2 associates with miRNA precursors, we used THO2:eGFP transgenic plants to immunoprecipitate the fusion protein and search for associated RNA molecules. After selecting plants by fluorescent microscopy, we pulled down the THO2:eGFP fusion using an anti-GFP antibody. Total or THO2-associated RNA was extracted from the input and immunoprecipitate fractions, and used to synthesize cDNA. RT-PCR experiments revealed that all tested miRNA precursors associate with THO2 (Figure 7b). We then evaluated whether the observed association is important for recruitment of miRNA precursors into the processing complex. In order to test this scenario, we evaluated the HYL1-associated miRNA precursors by using an anti-HYL1 antibody to co-immunoprecipitate the protein in the *tho2-5* mutant background. Interestingly, we found a reduction in the HYL1-associated miRNA precursors in *tho2-5* plants (Figure 7c,d) despite these mutants containing more pri/pre-miRNAs than wild-type plants (Figure 6f). The association of THO2 with miRNA precursors and the reduced association with HYL1 observed in the *tho2-5* background suggest a role for THO2 in transport of these RNA molecules to the processing complex or in their stabilization. Interestingly, the precursors of miR171a, which were not drastically reduced in *tho2* mutant plants (Figure 6d), appeared to be poorly associated with THO2 (Figure 7b). In *tho2*-5 mutants, the level of HYL1-associated miR171a precursors appeared unchanged compared to wild-type plants (Figure 7c,d), suggesting an alternative pathway for this miRNA.

THO2 is required for splicing in Arabidopsis plants

In humans, the THO/TREX complex is involved in splicing through interaction with the spliceosomes (Rappsilber *et al.*, 2002). In Arabidopsis, it has been shown that mutations in the *emu*/*tho1* locus affect the alternative splicing of genes encoding serine/arginine-rich (SR) proteins (Furumizu *et al.*, 2010). Supporting this reported role of the THO/TREX complex during mRNA splicing, we detected a partial overlap between the nuclear localization of THO2 and SRp34, a canonical spliceosome component (Figure 7e). Despite sharing localization in the same nuclear speckles, THO2 also localized in the cell nucleolus, a feature that is not shared with SRp34, suggesting additional roles of THO2. We also performed RT-PCR using mRNAs extracted from *tho2* seedlings to determine the splicing patterns of several SR genes encoding proteins involved in RNA splicing. Our analysis indicated that, of all tested SR genes, only *SR34b* showed a differential splicing pattern in all *tho2* alleles compared to wild-type plants (Figures 7f and S3a). To further examine the alternative splicing patterns of SR34b, we used capillary electrophoresis to analyze the splicing isoforms in samples from the *tho2-6* mutant. Double-stranded DNA derived from the splicing fragments was generated by RT-PCR and separated in a

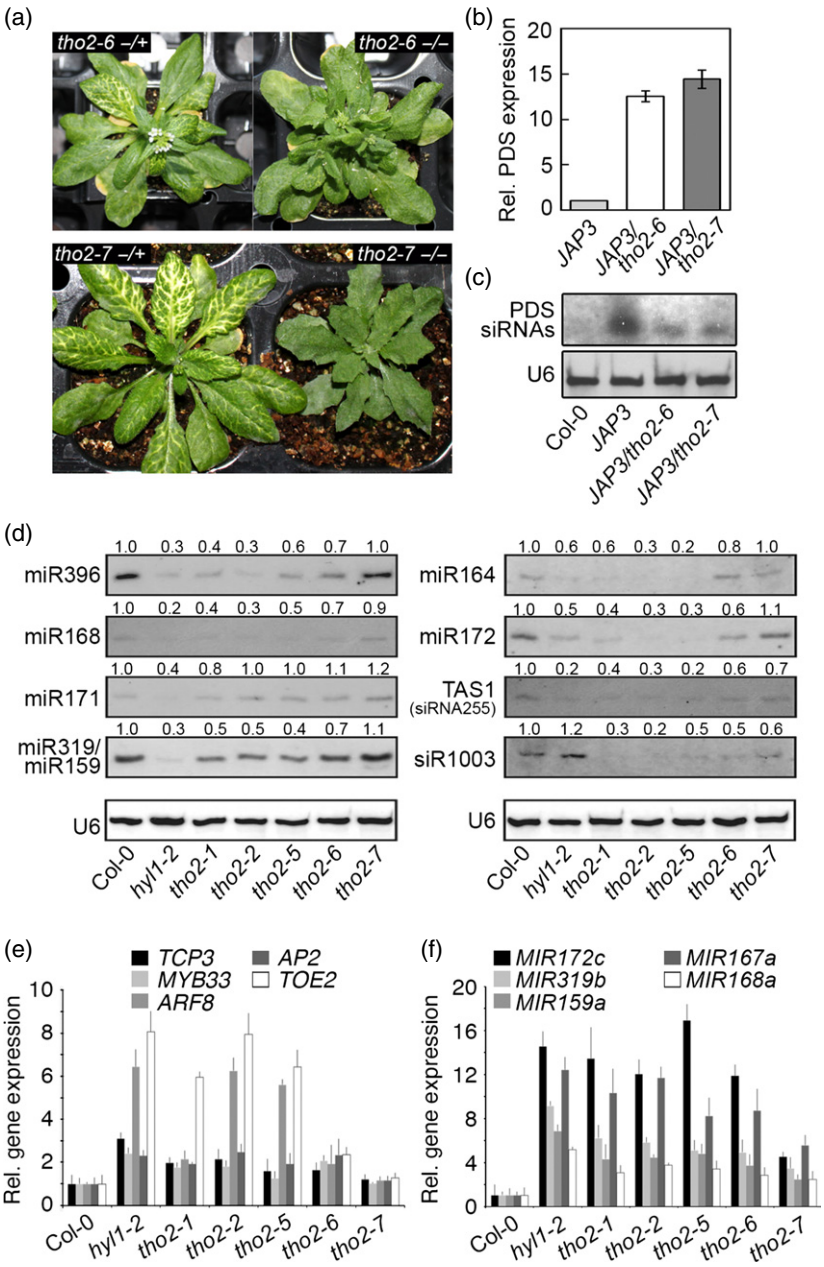

Figure 6. Effects of mutation in *THO2* on accumulation of small RNAs.
(a) Phenotypes of the *JAP3/tho2-6* and *JAP3/tho2-7* mutants.
(b) Quantitative RT-PCR analyses of *PDS* expression in *JAP3*, *JAP3/tho2-6* and *JAP3/tho2-7* plants. Error bars indicate 2 × standard error of the mean (SEM).
(c) RNA blots for *PDS*-derived siRNAs. The U6 small nuclear RNA (U6) was used as a loading control.
(d) RNA blots for detecting miRNAs, TAS1-derived tasiRNAs (siRNA 255) and siR1003. U6 was used as a loading control. The relative abundance of small RNAs was calculated by measuring the band intensity using ImageJ (http://imagej.nih.gov/ij/), and is indicated above each band.
(e, f) Quantitative RT-PCR analysis of miRNA targets and miRNA precursors. Error bars indicate 2 × standard error of the mean (SEM).

capillary electrophoresis device. The resulting pattern for each sample reflects the sizes of the observed splicing forms. The analysis confirmed the differential splicing pattern of *SR34b* in *tho2-6* mutants, clearly showing a shift to smaller transcript fragments in the mutant (Figure 7g). We did not find any difference in the splicing pattern of intron-containing or intron-contained miRNA genes between mutant and wild-type plants (Figure S3b). Given these findings, it is possible that aberrant splicing or mRNA destabilization of general small RNA factors may generate a reduction in miRNA biogenesis, contributing to the observed miRNA reduction. Alternatively, given the THO/TREX function in mRNA transport, it may be possible that the mRNAs of miRNA-related factors fail to reach the ribosomes, and thus are not translated. Quantitative RT-PCR analysis showed no significant difference in the mRNA accumulation of several miRNA factors (Figure S4a). We only detected a slight increase in *AGO1* mRNA in the mutant plants, probably a consequence of the reduction in miR168, thus discounting a mRNA destabilization scenario. RT-PCR analysis of the mRNAs of miRNA-related factors showed no differential splicing patterns between wild-type and mutant plants, at least for tested regions of the transcripts (Figure S4b). Such observations discount the possi-

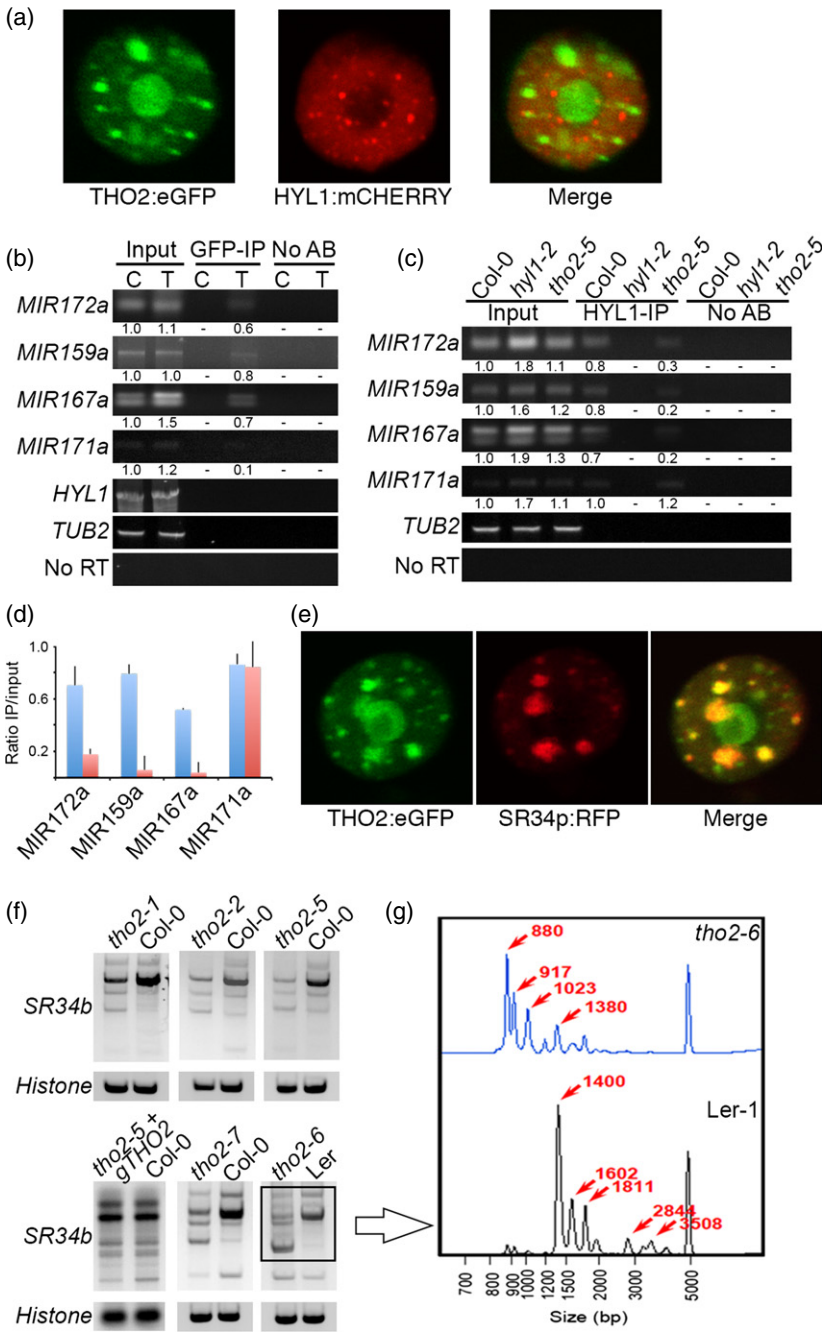

Figure 7. THO2 binds miRNA precursors, delivers them to the processing complex, and regulates mRNA splicing in Arabidopsis.
(a) Confocal microscopy images showing the nuclear localization of THO2:eGFP and HYL1:mCherry.
(b) RT-PCR to detect THO2-associated miRNA precursors in RNA immunoprecipitation samples. C, 3xGFP:NLS transgenic plants were used as a negative control for un-specific binding. T, THO2:eGFP transgenic plants. Lanes 5 and 6 ('No AB') show RT-PCR performed on samples to which no antibody was added during the RNA immunoprecipitation protocol. RT-PCR measurement of *HYL1* mRNA was performed to exclude un-specific binding of THO2. β-TUBULIN2 mRNA was used as a control mRNA. The 'No RT' control was performed using a mixture of primers amplifying the miR159a, 167a, 171a and 172a precursors. Band intensity was quantified using ImageJ, and is indicated below each band.
(c) RNA immunoprecipitation assay performed using an anti-HYL1 antibody.
(d) Quantitative RT-PCR analysis of the RNA immunoprecipitation miRNA precursors. Data are expressed as a ratio between the immunoprecipitate and input fractions for Col-0 plants (blue bars) and *tho2-5* mutants (red bars). Expression levels were normalized against the levels of β-TUBULIN2 (At5g62690) in the input fraction. Error bars indicate 2 × standard error of the mean (SEM).
(e) Confocal microscopy images showing partial co-localization of THO2:eGFP and SR34p:RFP.
(f) RT-PCR analysis of splicing patterns of *SR34b* in wild-type, *tho2* mutants and *tho2-5* complemented mutants (*tho2-5*+ gTHO2). Histone was used as a loading control.
(g) Splicing isoforms of *SR34b* generated in *tho2-6* and Col-0 were analyzed via capillary electrophoresis. Various sizes of isoforms (in bp) are indicated.

bility that aberrant splicing of miRNA biogenesis factors contributes to the reduction in their production. Finally, Western blotting using antibodies against HYL1, AGO1 and DCL1 revealed no significant change in the accumulation of these proteins in the mutant plants (Figure S4c). These results indicate that, at least for these miRNA-related genes, mRNA translation is not affected in the mutants. The observed over-accumulation of AGO1 in the *tho2-5* mutant may potentially reflect loss of the miR168–AGO1 regulation node.

ABA HYPERSENSITIVE 1 (ABH1), CAP-BINDING PROTEIN 20 (CBP20) and DAWDLE (DDL) are miRNA biogenesis co-factors involved in mRNA splicing and miRNA precursor stabilization (Kim *et al.*, 2008; Laubinger *et al.*, 2008; Yu *et al.*, 2008). The overlap between these proteins and THO2 functions led us to speculate whether THO2 interacts with them. However, our yeast two-hybrid results indicated that THO2 does not interact, at least directly, with ABH1, CBP20 or DDL (Figure S5). In any case, these results do not exclude the possibility that the THO/TREX complex, once fully assembly, interacts with these proteins to fulfill its miRNA-related functions.

DISCUSSION

The THO/TREX complex is highly conserved in multiple organisms, acting in various contexts. In humans, it is a key component of the mRNA export machinery, and appears to be primarily associated with spliced mRNA (Masuda *et al.*, 2005; Chi *et al.*, 2013). THO2 is the largest protein of the complex, and is considered to be its core (Pena *et al.*, 2012). Based on the fact that *THO2* null mutants in Arabidopsis die at the embryonic stage, it has been proposed that the protein is essential for assembly of the complex and its function, and therefore for the survival of the plant (Furumizu *et al.*, 2010; Jauvion *et al.*, 2010; Yelina *et al.*, 2010). The isolation of viable *tho2* alleles, described in the present work, is a major breakthrough for study of the THO/TREX complex. Despite developmental defects, ranging from abnormal floral organs and leaf serrations to partial embryo lethality, observed in the newly characterized *tho2-5*, *tho2-6* and *tho2-7*, these mutants represent a unique genetic tool for studying THO2, the core protein of the THO/TREX complex (Pena *et al.*, 2012).

As observed in mutant plants for other components of the THO/TREX complex, *tho2* mutants show reduced siRNA-mediated silencing, with a concomitant reduction in the steady-state levels of siRNAs and tasiRNAs. We also observed reduced levels of mature miRNAs and over-accumulation of miRNA-targeted mRNAs in the *tho2* mutant plants (Figure 6). Previous reports have shown that other components of the complex, such as THO1, regulate miRNA accumulation (Furumizu *et al.*, 2010). However, TEX1, which is required for siRNA and tasiRNA production, does not appear to be involved in the miRNA pathway (Yelina *et al.*, 2010). Our data show that the *tho2* mutants present morphological and molecular phenotypes more severe than those observed in mutants for other THO/TREX components (Furumizu *et al.*, 2010; Jauvion *et al.*, 2010; Yelina *et al.*, 2010). This observation suggests that THO2 is central for the functionality of the complex, while each accessory protein may have a more specific function. This is supported by the extreme phenotype of the *tho2* mutants and the embryonic lethality of the *THO2* null alleles. It is probable that, in the absence of THO2, but not of the other components, the whole complex fails to assemble. However, more experimentation is required to confirm this. Interestingly, the *tho2-7* mutation, which generates a truncated messenger lacking the very last portion of the gene, does not lead to any alteration in miRNA accumulation, but still induces reduction of tasiRNAs, siRNA silencing and alternative splicing similar to what is observed for the other alleles (Figures 6 and 7). This may indicate that the missing portion of THO2 is, either by itself or through an interacting partner, dispensable for the miRNA regulation but essential for the other small RNA molecules. A potential candidate in this last scenario is TEX1, as it has been described as not being required for miRNA accumulation, but its mutation drastically affects both the accumulation of tasiRNAs and SUC2p:PDS transgene siRNAs (Smith *et al.*, 2007; Yelina *et al.*, 2010). However, it has been reported in yeast that the C-terminal region of the THO2 protein, a poorly conserved region of the protein, interacts with nucleic acids but has little effect on the integrity of the complex (Pena *et al.*, 2012).

Despite many studies examining the function of the plant THO/TREX complex, it remains unclear how the complex acts in small RNA pathways. Using a yeast two-hybrid approach, we observed that THO2 did not interact with any of the tested miRNA processing components (Figures S1 and S5). This observation, together with the fact that THO2 is found in a different subcellular localization to the miRNA processing factors, suggests that the effect on this pathway is not at the miRNA processing level. This idea is in agreement with the possibility that THO2 acts in multiple small RNA pathways. It has been proposed that the THO/TREX complex is required for transporting siRNA/tasiRNA precursors to an unknown subcellular location for their processing (Yelina *et al.*, 2010). This is consistent with the known function of the complex in mRNA trafficking in fungi and metazoans (Reed and Cheng, 2005). In the present work, we show experimental evidence supporting such a scenario. We show that THO2 is able to interact with miRNA precursors, an interaction that appears to be important for recruitment of these molecules to the processing complex. The fact that *tho2* mutants fail to accumulate several classes of small RNAs may suggest that the complex has a broad affinity for a common component of all these pathways. Thus it is possible that the THO/TREX complex

recognizes and transports double-stranded RNA, which, despite the difference in their origin and nature, is a common feature of all small RNA precursors. Results obtained in previous reports (Furumizu *et al.*, 2010; Jauvion *et al.*, 2010; Yelina *et al.*, 2010) suggest that each individual component of the THO/TREX complex in plants, other than THO2, specifically affects a small RNA pathway, probably by differential preference for precursors.

In recent years, it has become evident that the miRNA pathway is linked or at least affected by the splicing machinery. Proteins such as SE, the cap-binding complex and TOUGH have been found to play roles in miRNA biogenesis and alternative splicing in Arabidopsis (Kim *et al.*, 2008; Laubinger *et al.*, 2008; Ren *et al.*, 2012). Our results revealed similar dual roles for THO2. Not surprisingly, the weak loss-of-function alleles of *tho2* display morphological defects similar to *se*, *cbp80*, and *cbp20* mutants, while both *se* and *tho2* null mutants die at embryonic stages (Hugouvieux *et al.*, 2001; Bezerra *et al.*, 2004; Papp *et al.*, 2004). We found that THO2 shared nuclear localization with the canonical splicing factor SR34p, and identified a case of abnormal alternative splicing in the *tho2* mutants. A genome-wide analysis of alternative splicing in plants mutated in each of the genes encoding components of the complex is required in order to understand how this process is affected by dysfunction of the complex.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *tho2-5* mutant was isolated in a previously described forward-genetics screen (Manavella *et al.*, 2012a). *tho2-1* (SALK_072011), *tho2-2* (SALK_130342) and *tho2-7* (SALK_144229) were obtained from the Arabidopsis Biological Research Center (https://abrc.osu.edu/). The *tho2-6* mutant was isolated from an *Arabidopsis thaliana* Landsberg *erecta* (L*er*) activation tagging screen performed using the pSKI015 vector as previously described (Weigel *et al.*, 2000). All seeds were sown on soil or MS plates, and were cold-treated in the dark for 3 days before transfer to growth chambers under long-day conditions (16 h light/8 h of dark) at 22°C/20°C (day/night). PCR primers used to genotype *tho2* alleles are listed in Table S1.

Transgenes

The miRNA–reporter transgenic lines (35S:Luc;35S:amiRLuc) and control lines (35S:Luc) have been described previously (Manavella *et al.*, 2012b). A triple GFP construct with nuclear localization signal (3xGFP:NLS) has been described previously (Mathieu *et al.*, 2007). THO2:eGFP and HYL1:mCHERRY constructs were obtained by RT-PCR-mediated cDNA amplification, cloning into pCR8GW-TOPO (Life Technologies, www.lifetechnologies.com), and recombined into modified pGREEN vectors under the control of the CaMV 35S promoter to generate C-terminal fusions with the fluorescent proteins. The SRp34:RFP construct has been described previously (Lorkovic *et al.*, 2004). The THO2 genomic construct (gTHO2), used to rescue *tho2-5* mutants, was generated by fusing the PCR-amplified *THO2* cDNA and a 2000 bp fragment upstream of the *THO2* transcription start site. The obtained product was cloned into pCR8GW-TOPO, and recombined into a modified pGREEN vector to generate a mCitrine C-terminal fusion. Yeast two-hybrid constructs were obtained by cloning the specific cDNAs into pCR8GW-TOPO, followed by recombination into the pDEST32 or pDEST22 vectors (Life Technologies). *Arabidopsis thaliana JAP3* and *tho2-5* mutant plants were transformed using the flower-dip method (Clough and Bent, 1998). Transgenic seedlings were selected using 50 mg ml^{-1} kanamycin on plates or 0.1% ammonium glufosinate on soil. At least 15 T_1 seedlings were analyzed for each construct. Transient infiltration of *Nicotiana benthamiana* leaves was performed as described previously (de Felippes and Weigel, 2010).

TAIL-PCR analysis

Genomic DNA samples were prepared from young leaves using a modified cetyl trimethyl ammonium bromide method (Murray and Thompson, 1980). To determine the sequences flanking the T-DNA insertion, we used nested pSKI015-specific primers (TR1, TR2, and TR3) and an arbitrary degenerate primer (P7) as described by Liu *et al.* (1995). The resulting PCR products were sequenced. The right border region was located using RB1, RB2 and RB3 primers. All primer sequences used are listed in Table S1.

Expression analysis

Total RNA was extracted using an RNeasy plant mini kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions. Primers for RT-PCR and quantitative RT-PCR were designed in the intron flanking regions. cDNA synthesis from 1 μg total RNA was performed using an ImpromII reverse transcription system kit (Promega, www.promega.com/), with an oligo(dT) primer, according to the manufacturer's instructions. Quantitative RT-PCR, small RNA gel blots, confocal microscopy and luciferase measurements were performed as previously described (Manavella *et al.*, 2012b). RNA blots to detect *PDS*-derived siRNAs were performed using a radioactively labeled PCR-amplified fragment of the *PDS* gene as probe. Primers used for quantitative PCR and as RNA probes are listed in Table S1. Western blot analysis was performed using anti-HYL1, anti-DCL1 and anti-AGO antibodies (Agrisera, www.agrisera.com/) as previously described (Manavella *et al.*, 2013).

Yeast two-hybrid assays and confocal microscopy

Yeast two-hybrid assays were performed using the ProQuest™ two-hybrid system (Life Technologies), according to the manufacturer's instructions. To reduce auto-activation of THO2, we added 40–120 mM 3-amino-1,2,4-triazole to the selection medium. For the microscopy experiments, *N. benthamiana* leaves were transiently co-transformed with a THO2:eGFP fusion and a HYL1:mCherry or SR34p:RFP fusion (de Felippes and Weigel, 2010), and imaged using a TCS SP2 confocal microscope (Leica, http://www.leicamicrosystems.com/) on day 3 after infiltration.

RNA immunoprecipitation assay

RNA immunoprecipitation experiments were performed as previously described (Terzi and Simpson, 2009). Fifteen-day-old THO2:eGFP, 3xGFP:NLS, Col-0, *hyl1-2* and *tho2-5* plants were grown on MS agar plates. An anti-GFP antibody (Abcam, www.abcam.com), an anti-HYL1 antibody (Agrisera) and Protein G–agarose beads (Life Technologies) were used to immunoprecipitate protein–RNA complexes. After elution of protein–RNA complexes, RNA and proteins were extracted using TriPure reagent (Roche, http://

www.roche.com). First-strand cDNA and RT-PCR of the associated RNAs were performed as described above.

Splicing fragment analysis

RT-PCR fragments were analyzed using a Fragment Analyzer™ automated CE system (Advanced Analytical Technologies, http://www.aati-us.com/). Diluted samples (1/10) were prepared using a DNF-910/15L80M dsDNA reagent kit (Advanced Analytical Technologies), according to the manufacturer's instructions. Separation results were analyzed using PROSize® 2.0 software (Advanced Analytical Technologies).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. miRNA accumulation in *tho2-5* complemented plants.

Figure S2. Yeast two-hybrid assay for testing the interaction of THO2 with a collection of miRNA-related factors.

Figure S3. Splicing patterns in *tho2* mutants.

Figure S4. Splicing patterns, mRNA and protein accumulation of miRNA biogenesis factors in *tho2* mutants.

Figure S5. Yeast two-hybrid assays for the interaction between THO2 and DDL, ABH1 or CBP20.

Table S1. Oligonucleotide sequences used in this study.

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Supporting Information

THO2, core member of the THO/TREX complex, is required for regulation of small RNA abundance in Arabidopsis

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SUPPLEMENTARY FIGURES

Supplementary Figure 1. Small RNA accumulation in *tho2-5* complemented mutants and gTHO2:mCitrine sub-cellular location.

(a) RNA blots for detecting miRNAs, TAS1-derived tasiRNAs (siRNA 255), and siR1003. U6 was used as loading control. Relative small RNA abundance was calculated by measuring the band intensity with ImageJ and noted over each band. A combination of the *THO2* promoter with the THO2 coding sequence fused to the fluorescent protein mCitrine (gTHO2) was used to confirm the role of THO2 in the small RNA production.

(b) Confocal microscopy images showing the nuclear localization of gTHO2:mCitrine. The chlorophyll auto-fluorescence appears in red.

Supplementary Figure 2. THO2 Y2H interaction screen.

Yeast transformants were plated on selective medium without leucine and tryptophan (-LT), or without leucine, tryptophan and histidine (-LTH). All miRNA-related factors were cloned fused to the GAL4 activation domain (AD) while THO2 to the GAL4 binding domain (BD). Serial dilutions of the yeast were plated to ensure even initial yeast density. The known interaction between SERRATE (SE) and HYL1 was used as a positive control.

Supplementary Figure 3. mRNA splicing in *tho2* mutants.

(a) RT-PCR analysis of splicing patterns of *SR1, SR40, SR45* and *SR34a* in wild type and *tho2* mutants. Histone was used as loading control.

(b) Splicing pattern of MIRNA genes analyzed by RT-PCR. PCRs, using wild type genomic DNA (gDNA), were performed as amplification controls.

Supplementary Figure 4. Expression and splicing of miRNA-related factors in *tho2-5* mutants.

(a) qRT-PCR analysis of miRNA-biogenesis factors in wild type and *tho2-5* plants. Error bars indicate 2 x standard error of the mean (SEM).

(b) mRNA splicing pattern of MiRNA-related genes analyzed by RT-PCR. Primers used for the analysis expand the full-length coding region (HYL1), exons 7-12 (SERRATE), exons 14-20 (DCL1), exons 2-10 (AGO1a) and exons 13-22 (AGO1b). PCRs, using wild type Col-0 genomic DNA (gDNA), were performed as amplification controls.

(c) AGO1, HYL1 and DCL1 protein accumulation in wild-type Col-0, *tho2-5* mutant and *tho2-5* compensated mutant plants as detected by western blot. Proteins extracted from *ago1-36, hyl1-2* and

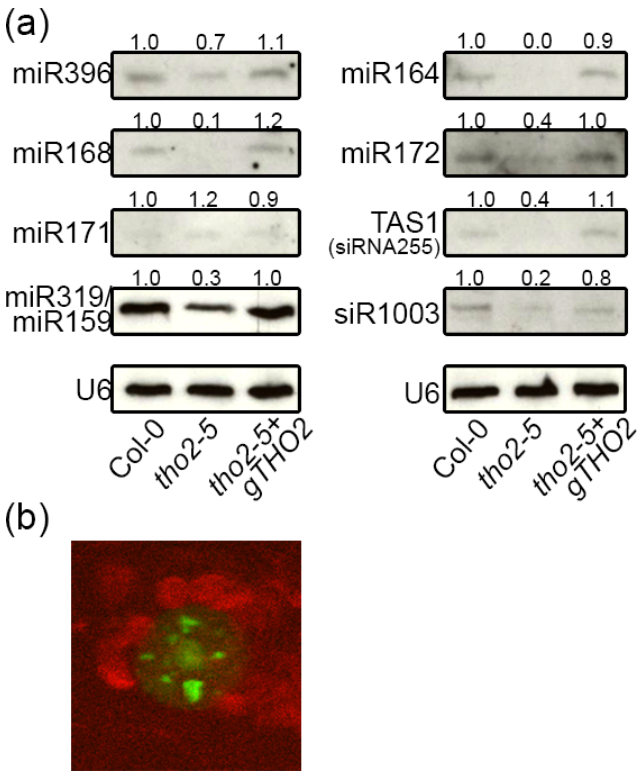
dcl1-100 mutants were used as antibody specificity controls. Coomassie staining is shown in the bottom of each blot as loading control.

Supplementary Figure 5. THO2-DAWDLE, -ABH1 and –CBP20 Y2H interaction.
Yeast transformants were plated on selective medium without leucine and tryptophan (-LT), or without leucine, tryptophan and histidine (-LTH). THO2, DAWDLE (DDL), CAP-BINDING PROTEIN 20 (CBP20) and ABA HYPERSENSITIVE 1 (ABH1) were fused to the GAL4 activation domain (AD) and GAL4 binding domain (BD). Serial dilutions of the yeast were plated to ensure even initial yeast density. The known interaction between SERRATE (SE) and HYL1 was used as a positive control.

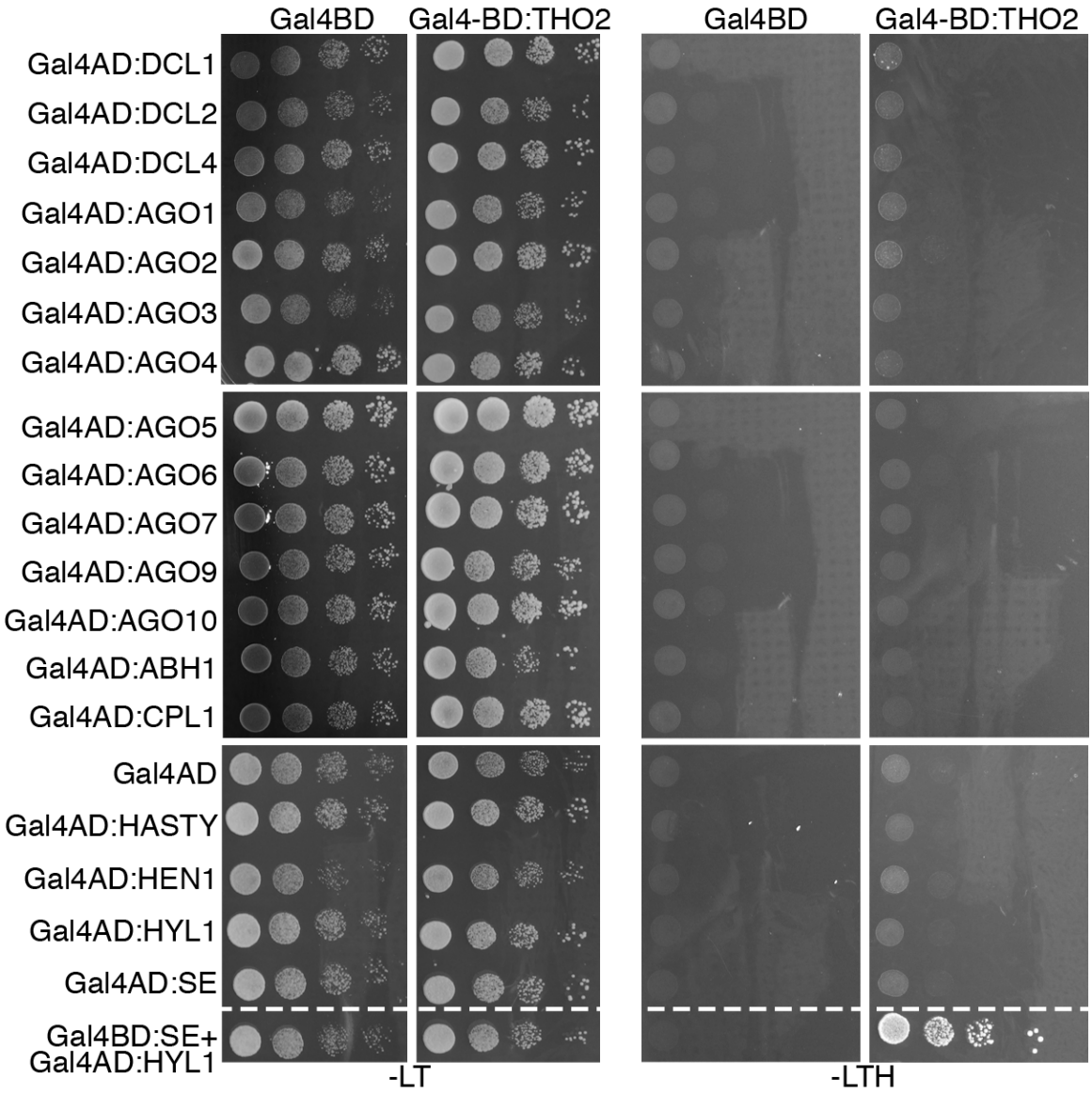
SUPPLEMENTARY TABLES

Supplementary Table 1. DNA oligonucleotide primers and probes.

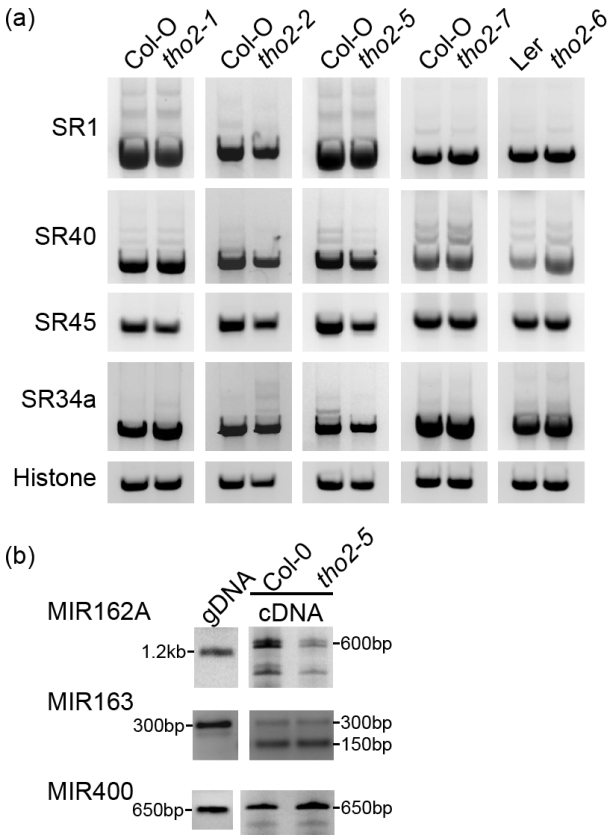
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



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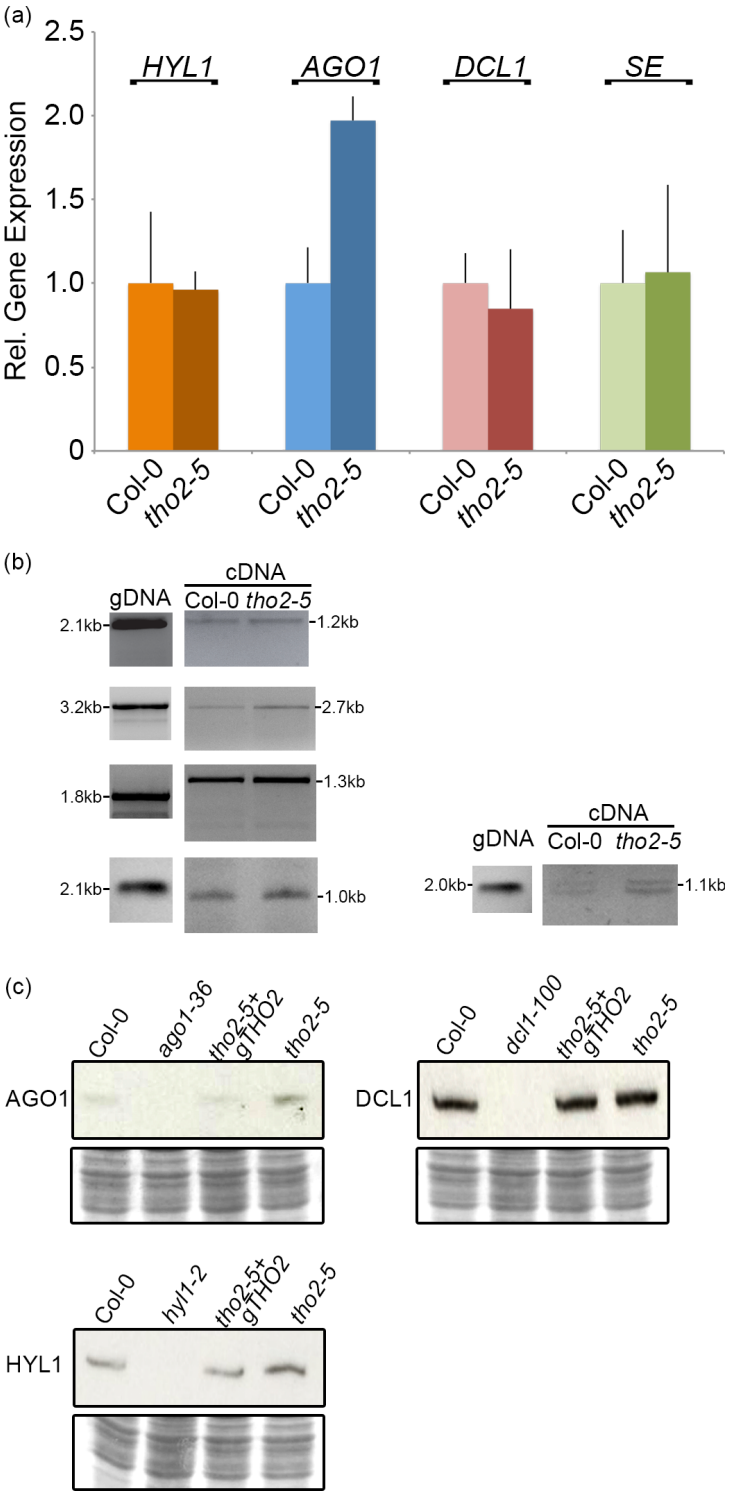
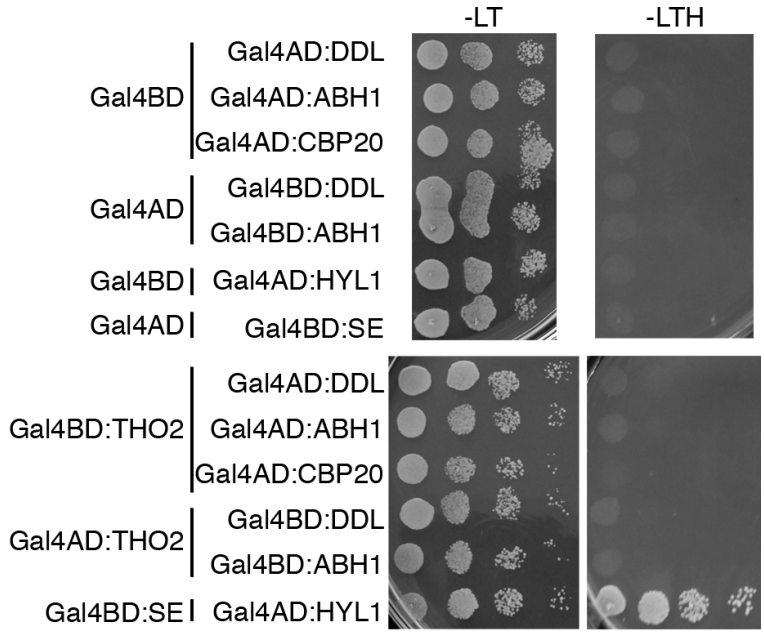


Supplementary Figure 5.



Supplementary Table 1.

Oligonucleotides	Oligonucleotide Sequence (5' to 3')	Purpose
TR1	TTGCTTTCGCCTATAAATACGAC	TAIL-PCR
TR2	ATAATAACGCTGCGGACATCTAC	TAIL-PCR
TR3	CTTTCTTTTCCTCCATATTGACC	TAIL-PCR
P7	GWWGGTSCWASWCTG	TAIL-PCR
RB1	ACTCACTATAGGGCGAATTGGAGC	TAIL-PCR
RB2	CTATCGTTCAAGATGCCTCTACCG	TAIL-PCR
RB3	GACGTTCCAACCACGTCTTCAAAG	TAIL-PCR
THO2	F: CCGCTACTGGAGTGTAAGTACGTG R: GGACTGTCTGTGATTCCACAAGCC	RT-PCR, Genotyping
T-DNA-F	GCGTGGACCGCTTGCTGCAACT	Genotyping
Primers-1	F: CCGCTACTGGAGTGTAAGTACGTG R: GGACTGTCTGTGATTCCACAAGCC	RT-PCR
Primers-2	F: TTAATGGTTTCCTCTCTGTGG R: CTGTGTCAAGTACTTGAAGGC	RT-PCR
Primers-3	F: CAACATCATTTGGCATGATGCG R: TACATATCAGGACATCAATGTGG	RT-PCR
Primers-4	F: AAGGATACATGGTTGAGCTCC R: ATAATAAGGGCATTACGAATCTCC	RT-PCR
Primers-5	F: TGACGAGAATCCTGTCAAACC R: CTATCATAACTCCTAGTTGTTCC	RT-PCR
SR1/SR34	F: TCGACGACCAACAGAATGAG R: GCTAGGGCTCTTGCTTCCTT	RT-PCR
SR34b	F: GGCGATATCCGTGAAAGAGA R: TCTTCCAACAGACCCAGCTT	RT-PCR
RS31	F: GGAACGGTTGTTCGACAAGT R: GGACTTGGACGCCTACGATA	RT-PCR
SR30	F: CGCAAGTGTGAGGTTGAAGA R: ATGCAGCCGAGACAGAGTTT	RT-PCR
SR34a	F: TTGGCTTCAGACCAAATCTTC R: TTCTTTTGGCCATTTTCACC	RT-PCR
SR30	F: CGCAAGTGTGAGGTTGAAGA R: ATGCAGCCGAGACAGAGTTT	RT-PCR
RS40	F: ACTACGCCTGCCAAAATCAT R: CACCATCATACCCACCATCA	RT-PCR
SR45	F: TGACGCTGAGAAAGATGGTG R: CCTTCTTCGAACAGGACTGC	RT-PCR
Histone H3	F: TTGGAAGAAACAATGGCTCGTACC R: AAGCTTAAGCACGTTCTCCACG	RT-PCR
JAP RAGE LB3	CCGGCATGCAAGCTGATATC	T-DNA genotyping
PDSa	F: TCAGCGGCCGCTTTGTATGCCAGTAGTGGATCATA R: CTAGTCGACGAGTTTCTCAAGTTCTTTCATTGTTG	RT-PCR
PDSb	F: GAACAACGAGATGCTGACATG R: TTCCAGGGATCTGGTAAAAGGAG	qRT-PCR
Actin	F: GCCATCCAAGCTGTTCTCTC R: GAACCACCGATCCAGACACT	qRT-PCR
miR396	CAGTTCAAGAAAGCTGTGGAA	RNA blot Probe
miR168	TTCCCGACCTGCACCAAGCGA	RNA blot Probe
miR171	GATATTGGCGCGGCTCAATCA	RNA blot Probe
miR319/159	AGGGAGCTCCCTTCAGTCCAA	RNA blot Probe
miR164	TGCACGTGCCCTGCTTCTCCA	RNA blot Probe
miR172	ATGCAGCATCATCAAGATTCT	RNA blot Probe
siRNA255	TACGCTATGTTGGACTTAGAA	RNA blot Probe
siR1003	ATGCCAAGTTTGGCCTCACGGTCT	RNA blot Probe
U6	GCTAATCTTCTCTGTATCGTTCC	RNA blot Probe
PDS siRNAs	F: TCAGCGGCCGCTTTGTATGCCAGTAGTGGATCATA R: CTAGTCGACGAGTTTCTCAAGTTCTTTCATTGTTG	RNA blot Probe
Pre-miR159a	F: GGTCTTTACAGTTTGCTTATG R: AGAAGGTGAAAGAAGATGTAG	qRT-PCR
Pre-miR319b	F: GGTCCACTCATGGAGTAATATGTG R: AGGGAGCTCCCTTCAGTCCAAGC	qRT-PCR
Pre-miR172c	F: CTGTTGGAGCATCATCAAGATTC R: AGCCACTGATTGCAGCTGCA	qRT-PCR
Pre-miR167a	F: TGAAGCTGCCAGCATGATCT	qRT-PCR

	R: AACGGGTGAAACTGCGAACA	
Pre-miR168a	F: CACCATCGGGCTCGGATTCGC R: AGTTGATGCAAGGCGGGATCC	qRT-PCR
TCP3	F: AGGGATGATGATGGTGGAGA R: CGGAGGATTTGTGTTTGCTT	qRT-PCR
MYB33	F: GAGTTTCATCTGCATTTTGTGTG R: TCCCTTCATTCCAATATTCAG	qRT-PCR
ARF8	F: CCACCACTGCCTTCTCCA R: TTCAGCAGCTACCACGAGCTG	qRT-PCR
AP2	F: TACACGTACTTCGCCGACAA R: GGTGTCGAACAAACCCAAAT	qRT-PCR
TOE2	F: ACTGGACTGATCATGCCCTT R: ATGGAGAACCACATGGCTG	qRT-PCR
HYL1	F: GTGCCAGAAGGTCGAAACTC R: CAGTTCTCCCAGCGCTAATC	qRT-PCR
DCL1	F: TCAAGTAGAAGAACCGCAGCTG R: AAGAGATTTACGTTTGGGGTAAGAG	qRT-PCR
SERRATE	F: CACAGAAGGTGGCAAAGGAT R: CGACAAGCTCCTGTAATCAATAAC	qRT-PCR
AGO1	F: TCAGCAGTAGAACATGACACG R: TCGGTGGACAGAAGTGGGAATA	qRT-PCR
BETA-TUBULIN2	F: GAGCCTTACAACGCTACTCTGTCTGTC R: ACACCAGACATAGTAGCAGAAATCAAG	qRT-PCR
MIR162A	F: GAGAGAGGAGGGATGTAGTAGGC R: GCAGCTCAAGGCATGGCAGA	RT-PCR
MIR163	F: GGTTGTGCCTGAAAATATGGGTTTC R: CACAACCATTTGCATTCCTTCGGAGG	RT-PCR
MIR400	F: CCGAATCAGCTTGAAGCAAAAATCG R: TAGTGAATCATAGGGTTCCACC	RT-PCR
AGO1a	F: CTTTGCTGAACTGCCTGATAAGG R: AGCATAATCATTGAGTTGCACCG	RT-PCR
AGO1b	F: GATCTGCTTATTGTCATTCTGCCCG R: TCAGCAGTAGAACATGACACG	RT-PCR
DCL1	F: GATGTTGAACCCTCCACGAC R: TCAAGAAAAAGTTTTATTTAAAAGCTC	RT-PCR
HYL1	F: ATGACCTCCACTGATGTTTC R: CAAGCTCCTGTAATCAATAAC	RT-PCR
SERRATE	F: CACAGAAGGTGGCAAAGGAT R: CAAGCTCCTGTAATCAATAAC	RT-PCR

II

KH domain protein RCF3 is a tissue-biased regulator of the plant miRNA biogenesis cofactor HYL1

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The biogenesis of microRNAs (miRNAs), which regulate mRNA abundance through posttranscriptional silencing, comprises multiple well-orchestrated processing steps. We have identified the *Arabidopsis thaliana* K homology (KH) domain protein REGULATOR OF CBF GENE EXPRESSION 3 (RCF3) as a cofactor affecting miRNA biogenesis in specific plant tissues. MiRNA and miRNA-target levels were reduced in apex-enriched samples of *rcf3* mutants, but not in other tissues. Mechanistically, RCF3 affects miRNA biogenesis through nuclear interactions with the phosphatases C-TERMINAL DOMAIN PHOSPHATASE-LIKE1 and 2 (CPL1 and CPL2). These interactions are essential to regulate the phosphorylation status, and thus the activity, of the double-stranded RNA binding protein and DICER-LIKE1 (DCL1) cofactor HYPONASTIC LEAVES1 (HYL1).

micro RNA biogenesis | *Arabidopsis thaliana* | HYL1 | phosphorylation | gene silencing

Micro RNAs (miRNAs) are short 21- to 24-nucleotide (nt)-long single-stranded RNAs that play an important role in posttranscriptional gene regulation in many multicellular organisms. In plants, after transcription of an *MIRNA* gene by RNA polymerase II, the primary miRNA (pri-miRNA) is incorporated into nuclear bodies known as D-bodies (1). There, it undergoes a two-step maturation process orchestrated by the ribonuclease DICER-LIKE1 (DCL1) (2). In a first step, DCL1, aided by cofactors including the C2H2-zinc finger protein SERRATE (SE), the nuclear cap-binding complex (CBC), the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1), and the HYL1 phosphatase C-TERMINAL DOMAIN PHOSPHATASE-LIKE1 (CPL1), removes the two single-stranded RNA tails of the pri-miRNAs to form a largely double-stranded miRNA precursor (premiRNA) (2–5). For accurate excision of the final miRNA:miRNA* duplex and subsequent sorting of the active strand into specific effector complexes, the interaction between HYL1, SE, and DCL1 is crucial. In this context, it has been shown that dephosphorylation of HYL1 by CPL1 is important for correct processing and strand selection of the mature miRNAs (3). In the cytoplasm, the miRNAs associate with an ARGONAUTE (AGO) protein to form the active RNA-induced silencing complex (RISC). Guided by the sequence complementarity of the miRNA to its target, the RISC either induces cleavage or inhibits the translation of target mRNAs (6).

Mutations in genes encoding plant miRNA-related proteins cause a broad range of phenotypes, from embryo lethality of *dcl1* and *se* null mutants to various developmental and physiological defects in *hyl1, ago1*, and *hen1* mutants (4, 7–9). It is unclear how much of this variation in phenotypes reflects genetic redundancy, different processing requirements for different miRNA precursors, or tissue- and stage-specific activity of miRNA-related proteins. In animal systems, several protein complexes and cofactors that regulate different steps of miRNA biogenesis and function have been isolated and characterized (10). Only recently, an increasing number of miRNA biogenesis cofactors have been identified in plants. Most of the specific regulatory events controlled by these cofactors are not yet fully understood (3, 5, 11–16).

A number of genetic screens have been carried out to fill in the remaining blanks in the plant miRNA biogenesis pathway (3, 17–19). We have used an assay that exploits silencing of luciferase by an artificial miRNA as a reporter for miRNA activity to identify candidates for factors affecting miRNA biogenesis or function (3). Among the isolated mutants were two new alleles of *REGULATOR OF CBF GENE EXPRESSION 3* (*RCF3*), also known as *SHINY1* and *HIGH OSMOTIC STRESS GENE EXPRESSION 5* (*HOS5*). *RCF3* encodes one of the 26 K-homology (KH) domain proteins in *Arabidopsis thaliana*. KH domains are also found in heterogeneous nuclear ribonucleoprotein K (hnRNP K) and poly-r(C)-binding proteins (PCBPs) and are predicted to bind RNA (20–22). *RCF3* contributes to tolerance against various stresses, including low temperature and osmotic stress and response to the stress hormone abscisic acid (21–23). RCF3 and CPL1 both interact with the splicing factors RS40 and RS41, and *rcf3* mutants show aberrant intron retention (21). In association with CPL1, RCF3 also seems to inhibit transcription of a number of genes by preventing mRNA capping and thereby disabling the transition from transcription initiation to elongation (22).

Significance

Micro RNAs (miRNAs) are small RNA molecules that regulate gene expression posttranscriptionally in a process known as gene silencing. Fine-tuning the production of miRNAs is essential for correct silencing of their targets, which in turn is important for homeostasis and development. To fine-tune the production of miRNAs, plants deploy a combination of proteins that act as cofactors of the miRNA-processing machinery. Here, we describe REGULATOR OF CBF GENE EXPRESSION 3 (RCF3) as a tissue-specific regulator of miRNA biogenesis in plants. RCF3 interacts with the phosphatases C-TERMINAL DOMAIN PHOSPHATASE-LIKE1 and 2 (CPL1 and CPL2), ultimately affecting the phosphorylation of one of the main DICER-LIKE1 (DCL1) accessory proteins, HYPONASTIC LEAVES1 (HYL1), with a concomitant effect on miRNA production.

Author contributions: P.K. and P.A.M. designed research; P.K., M.D.C., H.W., F.K., and P.A.M. performed research; P.K., D.K.S., X.W., J.H., and P.A.M. analyzed data; and P.K. and P.A.M. wrote the paper.

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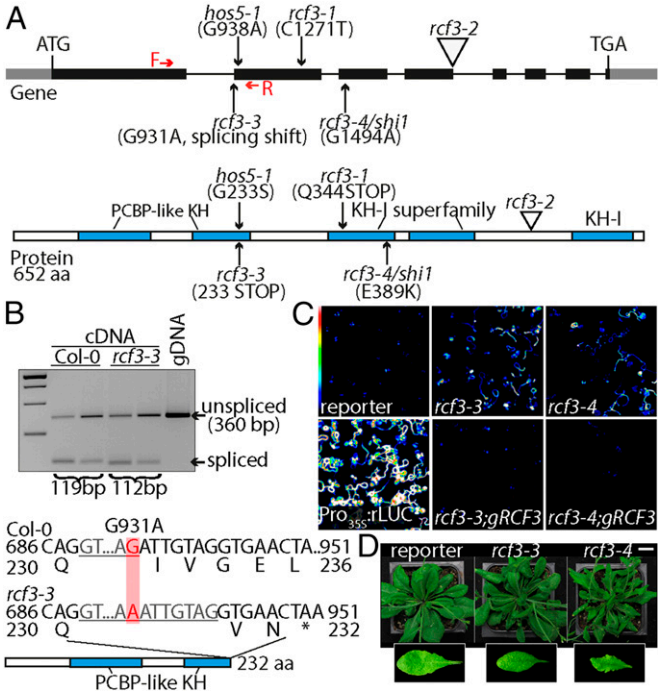

Fig. 1. Characterization of *rcf3* mutants. (*A*) Location and effects of mutations on the RCF3 protein. Red arrows note the forward and reverse primers used in *B* to detect the effect of *rcf3-3* mutation on splicing. The five KH domains are marked in blue. (*B*) RT-PCR analysis of *rcf3-3*, with genomic DNA (gDNA) for comparison. Sequencing revealed use of a cryptic splice acceptor site in the mutant, leading to a frame shift containing an early stop codon. (*C*) Bioluminescence phenotype of *rcf3* mutants, complemented mutants, amiRNA-activity reporter line (reporter), and *Pro35S:rLUC* controls. Colored scale indicates low (blue) to high (white) luminescence. (*D*) Morphological defects in *rcf3* mutants. (Scale bar: 2 cm.)

Here, we identify RCF3 as a regulator of CPL1-mediated HYL1 phosphorylation. We report that RCF3 localizes to similar nuclear speckles as other miRNA factors, including CPL1, DCL1, and SE. RCF3 interacts with CPL1 and its close homolog CPL2. Inactivation of RCF3 causes a shift of HYL1 phosphoisoforms toward the less active, hyperphosphorylated version. Accordingly, *rcf3* mutant defects can be corrected by overexpression of a hypophosphorylation mimic of HYL1. Unlike other known plant miRNA cofactors, RCF3 regulation of HYL1 phosphorylation and miRNA biogenesis takes place in specific niches in the regions spanning the vegetative and reproductive apices. Tissue-biased regulation of miRNA biogenesis is thus a phenomenon shared by plants and animals.

Results

Identification of Two *RCF3* Mutant Alleles as miRNA-Deficient Mutants. We identified several candidate genes required for miRNA biogenesis or function in a mutant screen using an artificial miRNA (amiRNA) that silences luciferase (3). Using whole genome sequencing and SHORE mapping (24), we mapped the locus responsible for apparent reduction of amiRNA activity, evident through reactivation of luciferase activity, in one of the isolated mutants to a region on chromosome 5 where we identified a mutation in the *RCF3* (At5g53060) gene (Fig. 1*A* and Fig. S1*A*). This allele, *rcf3-3*, contains a polymorphism that disrupts the first splice acceptor site in the middle of the sequence encoding the second of five KH domains (Fig. 1*A*). The mutation causes the splicing machinery to use an alternative cryptic acceptor site 7 nt downstream, as revealed by cloning and sequencing of mutant *RCF3* cDNA. This aberrant splicing leads to a frame shift and premature termination of translation (Fig. 1*B*). An additional allele isolated in our laboratory from an unrelated miRNA genetic screen, *rcf3-4*, contained a nonsynonymous substitution that affected the third KH domain (Fig. 1*A* and Fig. S1*A*). The exact same mutation has previously been identified in another screen (22). Transformation of the mutants with a genomic fragment of the WT *RCF3* locus restored silencing of the luciferase reporter and reverted the morphological phenotype in both *rcf3-3* and *rcf3-4*, confirming that the mutations in *RCF3* were the cause of the observed phenotypes (Fig. 1*C* and Fig. S1*B*). Although *rcf3-3* mutants have only subtle morphological defects, *rcf3-4* mutants were visibly impaired in growth and development, with elongated, rolled leaves and overall bushy growth (Fig. 1*D* and Fig. S1 *C* and *D*). The fact that *rcf3-4*, a missense allele, presents stronger defects than *rcf3-3*, a potential null mutant, suggests that redundantly acting factors might substitute for RCF3 when it is completely absent, as observed in other cases of unusual genetic redundancy (3). A database search for potential RCF3 homologs revealed that 12 of the 26 KH domain proteins encoded in the *A. thaliana* genome are closely related to RCF3 and are candidates for such redundant action (20) (Fig. S2).

***RCF3* Regulates miRNA Biogenesis Predominantly in Specific Plant Tissues.** To determine whether *RCF3* is required for miRNA biogenesis or for miRNA function, we evaluated the steady-state levels of mature miRNAs, miRNA precursors, and miRNA-targeted mRNAs in *rcf3* mutants. MiRNAs and their precursors, as well as miRNA-targeted mRNAs, seemed largely unaffected in whole 14-d-old seedlings, as determined by quantitative RT-PCR (RT-qPCR) (Fig. S3 *A*–*C*). MiRNA levels were also only mildly affected in rosette leaves of 25-d-old plants, as determined by RNA blots (Fig. S3*D*). Similarly, deep sequencing analysis of small RNAs extracted from 25-d-old leaves did not show significant differences in the abundance of miRNAs or miRNA*s between mutants and WT plants. Hierarchical clustering of genome-wide small RNA coverage profiles within 20 bases of either side of

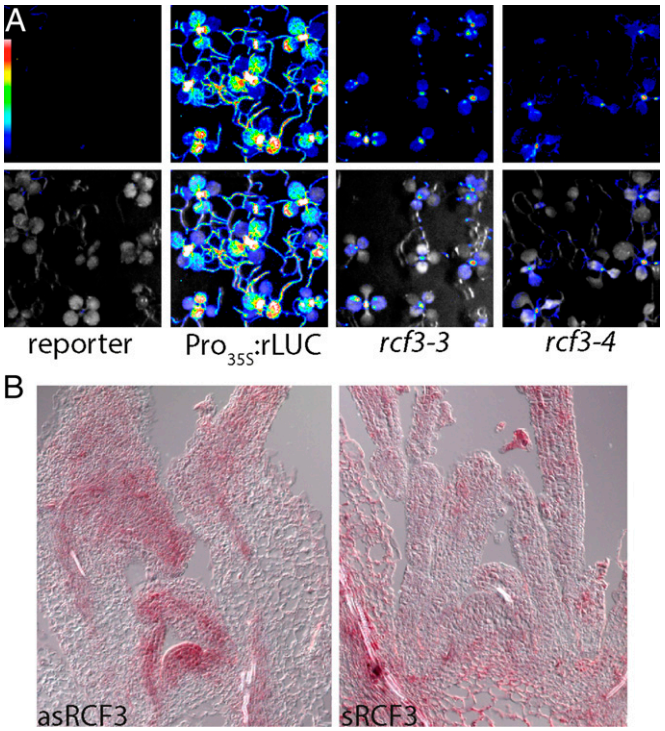

Fig. 2. *RCF3* expression and activity. (*A*) Bioluminescence activity in 10-d-old mutant plants, indicating preferential restoration of reporter activity in young tissue around the shoot apex. (*Top*) Luminescence. (*Bottom*) Luminescence merged with bright field image. Colored scale indicates low (blue) to high (white) luminescence. (*B*) Expression of *RCF3* visualized by in situ hybridization. (*Left*) Antisense probe. (*Right*) Sense probe.

mature miRNAs revealed only very subtle misprocessing of miRNA precursors in *rcf3*, albeit sufficient to allow mutant samples to cluster together (Fig. S3*E*). These observations were at first glance surprising, considering that the new *rcf3* mutant alleles were isolated from miRNA activity-reporting screens. A closer inspection of the mutant plants with a CCD camera revealed that the activity of luciferase was largely confined to the vegetative apical region (Fig. 2*A* and Fig. S4 *A* and *B*). This observation suggested that RCF3 might act, at least in the miRNA pathway, predominantly in this region of the plant. In agreement with this hypothesis, in situ hybridization showed that *RCF3* mRNA is abundant in the vegetative shoot apical meristem, young leaf primordia, and newly emerging leaves, a group of tissues hereinafter referred to as "vegetative apex" (Fig. 2*B*). Also in agreement with a spatially limited role of RCF3 in miRNA biogenesis, a clear reduction in the levels of several miRNAs was seen in very young (5- to 10-days-old) seedlings, which are enriched in vegetative apex tissue, whereas these differences largely disappeared in older plants (Fig. 3 *A* and *B*). The reduction in miRNA levels was paralleled by higher accumulation of several target mRNAs (Fig. 3*C*). The tissue-preferential effect of *RCF3* on miRNA accumulation and activity was confirmed with RNA isolated from 14-days-old vegetative apices, in which reduction in mature miRNAs and corresponding overaccumulation of miRNA-targeted mRNAs was much more evident (Fig. 3 *D*–F) than in age-matched whole-plant samples (Fig. S3 *A*, *B*, and *D*). Supporting the role of RCF3 in the miRNA pathway, a genome-wide analysis of small RNA levels in 14-days-old vegetative apices showed a clear reduction in miRNA accumulation without a detectable change in the abundance of other small RNAs (Fig. 4 *A* and *B*). This effect was particularly pronounced for highly expressed miRNAs, such as miR158a, miR166, miR159/319, and miR165 (Fig. 4*C* and Dataset S1).

We also observed, in the genome-wide small RNA analysis, an overaccumulation of miRNA* in *rcf3* mutants compared with WT vegetative apices, indicating a shift in miRNA precursor processing activity or altered AGO1 strand retention in *rcf3* mutants (Fig. 4*B*). A similar shift toward miRNA* accumulation has been described in *cpl1* and *hyl1* mutants, where misprocessing leads to altered miRNA/miRNA* ratios for a large portion of miRNAs. Our finding of a similar defect in *rcf3* mutants (Fig. 4*D*) suggests that RCF3 might act through CPL1 and HYL1, which would be in line with the reported protein–protein interactions between RCF3 and CPL1 and between CPL1 and HYL1 (3, 21, 22).

Available expression data for *RCF3* (25) report a peak of expression not only in the vegetative apex, but also in the reproductive apex, which we confirmed by RT-qPCR (Fig. S4*C*). We therefore monitored miRNA accumulation also in young *rcf3* inflorescences (samples, from here on referred to as "reproductive apex," included only young closed flower buds). Northern blot and RT-qPCR measurement of mature miRNAs revealed that *rcf3* mutant reproductive tissues accumulate lower levels of miRNAs, similarly to what we observed in vegetative apices (Fig. 3*G* and Fig. S4*D*). We also detected an overaccumulation of miRNA-targeted mRNAs and a particularly high reactivation of the amiRNA-activity reporter in the inflorescences (Fig. S4 *E* and *F*). Together, these findings suggest that RCF3, due to its predominant expression in the reproductive and vegetative apical regions, affects miRNA accumulation and activity unevenly across plant tissues. RT-qPCR revealed no significant change of pri-miRNAs and known miRNA-related factors in *rcf3* mutants, excluding that a potential transcription or splicing alteration was the cause of the observed phenotype (Fig. S5 *A* and *B*). However, we saw a slight overaccumulation of *CPL1* mRNAs in the mutant plants (Fig. S5*B*). Because this protein is a functional partner of RCF3 (this work and ref. 26), we speculate that the increase in transcript levels is probably a feedback response compensating for the reduction in RCF3 activity.

It has been previously shown that *RCF3* is also expressed in other tissues besides the vegetative and reproductive apices (21, 22). An analysis of the *RCF3* genomic location showed that the gene is located in a dense region of chromosome 5, with 11 genes in a 20-kb window. There are two natural antisense genes

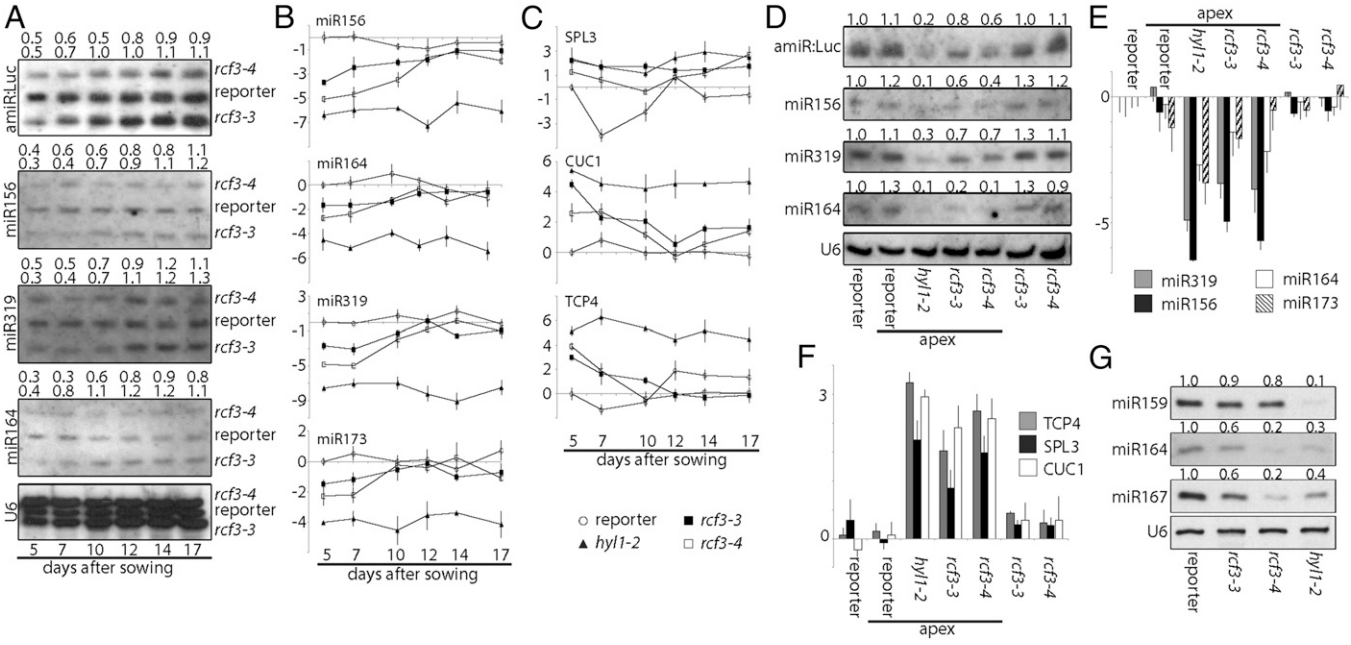

Fig. 3. miRNA levels and activity. (*A*) RNA blots for detection of mature endogenous miRNAs and amiRNA against luciferase (amiR:Luc) in a time course of 5- to 17-days-old whole seedlings. U6 was used as loading control. Samples were loaded on the same gel at 12-min intervals. Signal intensity was calculated with ImageJ and normalized to U6. Above each gel, the ratio of signal intensities of mutants to reporter control is noted. (*B* and *C*) Expression of mature miRNAs and miRNA targets as measured by RT-qPCR. Error bars indicate 2× SEM. The *y* axis shows the log2 of the relative expression. (*D*) RNA blots for detection of mature miRNAs and amiR:Luc in samples collected from 14-d-old whole plants or dissected vegetative apices. Expression relative to the reporter line is given on top. (*E* and *F*) Expression of mature miRNAs and miRNA targets as measured by RT-qPCR in the same samples as in *D*. Error bars indicate 2x SEM. The *y* axis shows the log2 values of relative expression. (*G*) RNA blots for detection of mature miRNAs in samples collected from inflorescences. Band intensity relative to the reporter line is given on top.

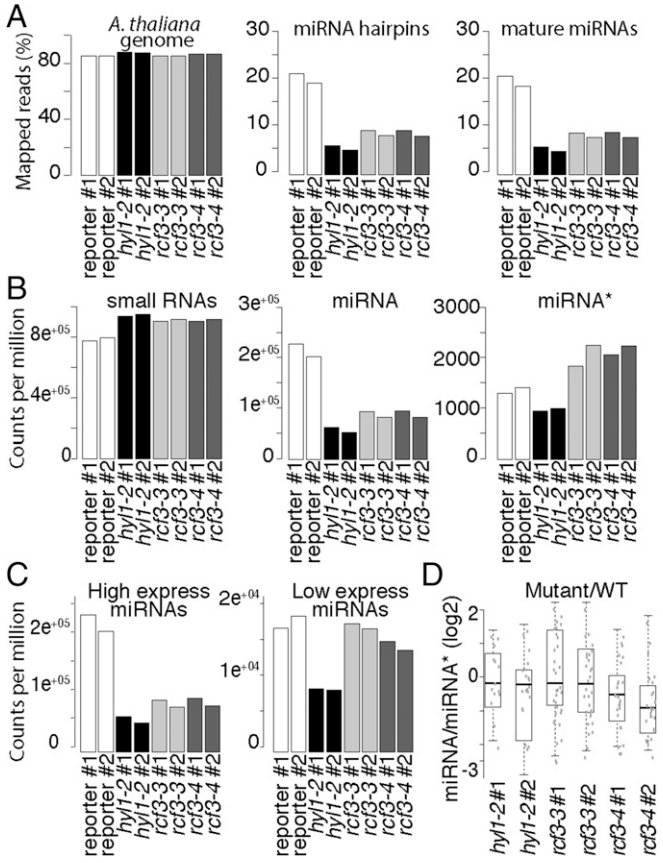

Fig. 4. sRNA, miRNA, and miRNA* levels in *rcf3* vegetative apices. (*A*) Fraction of reads mapping to the *A. thaliana* genome (TAIR 10), miRNA hairpins, and mature miRNAs. (*B*) Normalized counts per million mapped reads of all small RNAs, mature miRNAs, and miRNA*s. (*C*) Normalized counts per million mapped reads of highly and lowly expressed miRNAs. (*D*) Ratio of miRNA/miRNA* between mutants and control plants. For unique miRNAs (i.e., 156a-f), the ratio was calculated using the sum of all associated miRNA*s. The ratio was calculated only for those miRNAs that had greater than 2 counts per million in at least two samples. To compare the mutant to WT ratios, we first averaged the ratios of the WT replicates and then divided the mutant ratios by the mean WT ratio. The data were plotted as the log2 of the ratio mutant/WT showing misprocessed miRNAs as values below 0. Two biological replicates for each genotype: control miRNA-activity reporter line (reporter), *hyl1-2*, *rcf3-3*, *rcf3-4*.

(At5G53050 and At5G53048) located 3,175 bp upstream of the *RCF3* transcription start site (TSS) (Fig. S6*A*). Antisense genes can trigger transcriptional gene silencing (TGS) through small RNA-mediated DNA methylation. A bioinformatics search revealed the presence of DNA methylation marks and a peak of small RNAs mapping at ∼2,500 bp upstream of the *RCF3* TSS (Fig. S6*A*). Notably, in previous reports where a promoter:GUS fusion was generated to determine the *RCF3* expression pattern, the promoter fragments used did not extend into this particular region. To check whether the region influences the expression of *RCF3*, we cloned a larger fragment of the promoter. To our surprise, the new construct showed a far less promiscuous expression compared with the reported activity of the shorter constructs (Fig. S6*B*). Supporting the idea of *RCF3* being subject to transcriptional gene silencing (TGS), T1 plants carrying the reporter construct presented stronger GUS signal compared with their T2 counterparts. These data suggested a new layer of regulation on *RCF3* expression that will require furthers studies to dissect.

RCF3 Interacts with CPL1 and CPL2 to Regulate HYL1 Activity. Using transient expression of fluorescent-tagged fusion proteins, we found that RCF3 accumulated in nuclear speckles, where it colocalized with DCL1, SE, and CPL1, supporting a role for RCF3 as a miRNA biogenesis cofactor (Fig. 5*A*). To identify direct partners of RCF3 in the miRNA pathway, we performed a yeast two-hybrid (Y2H) interaction screen with RCF3 fused to GAL4-BD against a collection of 18 small RNA-related proteins. Among these proteins, RCF3 interacted only with CPL1 (Fig. 5*B* and Fig. S7*A*); this interaction has been previously reported (21, 22, 27) and confirmed *in planta* by bimolecular fluorescence complementation (Fig. S7*B*). RCF3 also interacted with CPL2 in yeast and plants (Fig. 5*C* and Fig. S7*B*), consistent with a partly redundant function of CPL1 and CPL2 (3). This interaction was not seen in another study (27), which, different from our experiments, did not use the full-length CPL2 protein. The interaction of RCF3 with CPL1/CPL2, together with the overaccumulation of miRNA* molecules in *rcf3* and *cpl1* mutants, suggested that RCF3 acts in the miRNA pathway through CPL1/2.

The miRNA biogenesis cofactor HYL1 is subject to phosphorylation, which regulates its activity (3, 28). HYL1 phosphorylation depends on CPL1, CPL2, and MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3). In *cpl1* mutant plants, HYL1 is hyperphosphorylated, and miRNA processing and strand selection are impaired (3). Because RCF3 interacted with CPL1 and CPL2, but not with HYL1, we tested whether lack of functional RCF3 had an indirect effect on HYL1 phosphorylation. The different

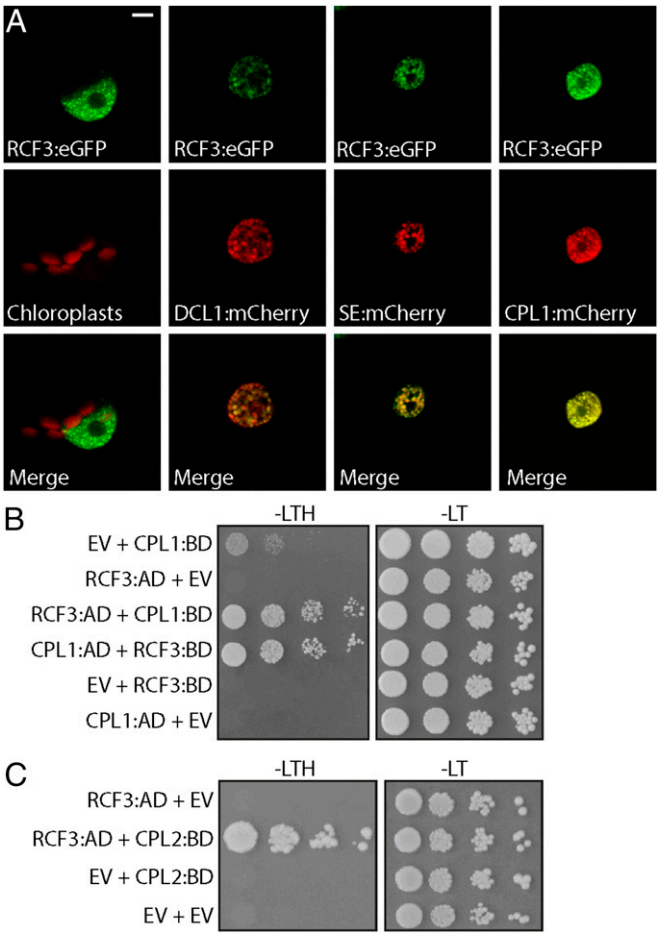

Fig. 5. Subcellular localization of RCF3. (*A*) Nuclear localization of RCF3:eGFP and colocalization with DCL1, SE, and CPL1 tagged with mCherry in transiently transformed *N. benthamiana* leaves. (Scale bar: 5 μm.) (*B* and *C*) Interaction of RCF3 with CPL1 and CPL2 in yeast two-hybrid assays. AD, GAL4 activation domain fusions; BD, GAL4 DNA binding domain fusions; EV, AD, or BD empty vectors; –LT, medium without leucine and tryptophan; –LTH, without leucine, tryptophan, and histidine. Shown are 1:10 serial dilutions.

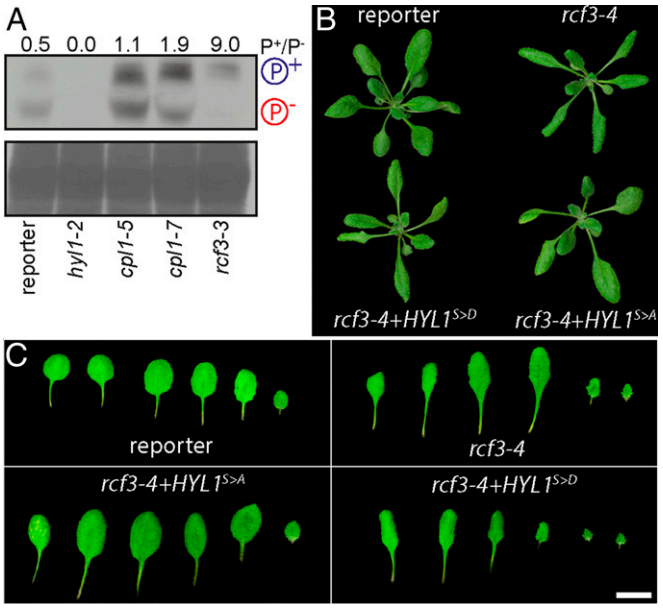

Fig. 6. Effect of RCF3 on HYL1 phosphorylation. (*A*) Phosphoprotein mobility shift assay, using PhosTag and anti-HYL1 antibodies. Hypo- and hyperphosphorylated HYL1 forms are indicated as P^- and P^+, respectively. Coomassie staining below as loading control. The intensity of the bands was measured with ImageJ and is expressed as the ratio of the hyper-/hypophosphorylated forms. (*B* and *C*) 14-days-old plants and leaf series, showing phenotypic rescue after transformation with HYL1 hyper- (S > D) and hypo- (S > A) phosphomimics. In *B*, plants were imaged individually and mounted in a single black background square to facilitate the comparison and observation.

phosphoisoforms of HYL1 were detected with the help of Phostag, a chemical compound that reduces the mobility of phosphorylated proteins in polyacrylamide gels. A strong shift toward hyperphosphorylated HYL1 was detected in 7-days-old *rcf3* mutant seedlings, even more so than in *cpl1* mutants (Fig. 6*A*). Identity of the HYL1 bands was verified with total protein samples extracted from *hyl1-2* mutants (Fig. 6*A*, lane 2). In agreement with RCF3 being primarily active in very young tissues, we did not detect any change in HYL1 phosphorylation in old plants or fully expanded leaves.

If excessive HYL1 phosphorylation contributes to the morphological defects in *rcf3* mutants, it should be possible to ameliorate these phenotypes by expressing a version of HYL1 that cannot be phosphorylated, mimicking the hypophosphorylated form of the protein. To test this hypothesis, *rcf3* mutants were transformed with two HYL1 phospho-mimics in which all potentially phosphorylated serines were mutated to aspartic acid (S > D) or alanine (S > A), to mimic either a hyper- or hypophosphorylated HYL1, respectively (3). Supporting a role of RCF3 in HYL1 phosphorylation, the morphological *rcf3* mutant defects were suppressed only in plants transformed with the hypophosphomimic (S > A) (Fig. 6 *B* and *C*). This rescue of the rosette leaf phenotype accords with our observation that RCF3 is highly expressed in the vegetative apex, where leaf shape is determined in leaf primordia.

Discussion

In animals, a series of proteins with specialized roles in miRNA biogenesis have been identified (10). In contrast, the differential contributions of individual proteins to miRNA biogenesis in plants are less well-understood even though mutations in these cofactors often cause distinct developmental and physiological defects. We have identified the KH domain protein RCF3 as a miRNA biogenesis cofactor that acts preferentially at the vegetative and reproductive apices. RCF3 promotes dephosphorylation of HYL1 (Fig. 6), likely through interaction with CPL1 and its homolog CPL2. In the vegetative apex, *rcf3* mutations seem to have a stronger effect on HYL1 phosphorylation than *cpl1* single mutations. This phenomenon might be explained by both CPL1 and CPL2 being subject to RCF3 action in this tissue, thus with *rcf3* mimicking *cpl1*/*cpl2* double mutants. A comparison of the HYL1 phosphorylation status detected in Fig. 6*A*, using young tissues, and in a previous report (3), where fully expanded leaves were used, suggests tissue-specific and possibly developmental stage-specific regulation of HYL1 activity by changes in its phosphorylation profile. An expression analysis using transcriptome datasets (25) revealed that, whereas *CPL2* is accumulated rather evenly across tissues (*CPL1* is not included in the analyzed datasets), *RCF3* is expressed most strongly in apices (Fig. S8). In a recent study, the protein kinase MPK3 was reported to trigger HYL1 phosphorylation, potentially antagonizing CPL1/2 function (28). Because *MPK3* transcripts are low in vegetative apices compared with other tissues, opposite to *RCF3* (Fig. S8), the shoot apex might be a niche where HYL1 activity, and therefore miRNA biogenesis, is particularly high because of high RCF3, but low MPK3 activity.

CPL1 and RCF3 negatively regulate the mRNA capping of several genes and play a role in splicing (22). Links between miRNA biogenesis, mRNA splicing, and capping activity have been established earlier for SE and the cap-binding complex (5, 29). These observations possibly position the CPL1–RCF3 complex close to SE and the cap-binding complex, suggesting that CPL1 and RCF3 might be recruited to pri-miRNA molecules early during their transcription. Even though RCF3 can interact with CPL1 and CPL2 (this work and refs. 30–34), its overall effects are more limited, at least partially because of the more restricted expression of *RCF3*. Simultaneous loss of *CPL1* and *CPL2* causes embryonic lethality whereas *rcf3*-null mutants have only minor developmental defects.

Integrating our data into the larger picture of miRNA biogenesis, we propose a hypothetical mode of action for RCF3 in the context of CPL1/2 and HYL1. Recently, it has been shown that DCL1 is recruited to pri-miRNAs during transcription (35). Due to their role in splicing, it is possible that additionally also SE and CPL1 are, like DCL1, among the first components to be recruited to the pri-miRNA during transcription. In the absence of CPL1, likely CPL2 will be incorporated instead (3). After this initial complex is formed, HYL1 and RCF3 might join the complex, a step dependent on both proteins' phosphorylation status, and anchor the precursors by specific RNA and protein interactions (3, 26). Both HYL1 and RCF3 are recruited to the complex in their hypophosphorylated isoforms: states that are reached due to the CPL1/2 phosphatase activity and antagonized by MPK3 (3, 26, 28). Localization experiments revealed that CPL1 activity is required for the correct localization of RCF3 and HYL1 (3, 21). The RCF3 mechanism of action, once the protein is recruited to the complex, remains unclear. We found that RCF3 affects the CPL1/2-mediated desphosphorylation of HYL1, but the specifics of this action are still unresolved. In this sense, *rcf3* mutants present normal CPL1 nuclear speckle localization (21), excluding the positivity of RCF3 being necessary for the CPL1 recruitment to the complex. One possibility is that RCF3 directly stimulates the enzymatic activity of CPL1/2 or that it serves as a bridge between CPL1 and HYL1. In any case, an extensive biochemical analysis will be required to dissect these possibilities.

Materials and Methods

Plant Material. *A. thaliana* accession Columbia (Col-0) or *Nicotiana benthamiana* seeds were surface sterilized with 10% (vol/vol) bleach and 0.5% SDS and stratified for 2–3 d at 4 °C. Plants were grown at 23 °C either on soil or on Murashige–Skoog (MS) plates (1/2 MS, 0.8% agar, pH 5.7) in long days (16 h light: 8 h dark). *hyl1-2* (N564863, SALK_064863), *cpl1*-7, and *rcf3-4* mutants have been described (3, 4, 22).

Transgenes. *RCF3* and *CPL2* coding regions were amplified by RT-PCR from RNA isolated from 10-days-old seedlings and cloned into pCR8GWTOPO. These constructs were used for Gateway-mediated recombination with ProQuest (Life Technologies) compatible vectors and pGREEN vectors. A detailed list of all constructs can be found in Table S1. The miRNA activity reporter (throughout the manuscript named "reporter"), the *CPL1*-fusion proteins, and the phosphomimic constructs have been described (3). As a positive luciferase control,

we mutated the amiRNA:luc target site in the luciferase cDNA to make it insensitive to the amiRNA-mediated silencing (PRO_{35S}:*rLUC*).

Mutant Screen and Luciferase Visualization. *rcf3* mutants were isolated and genetically mapped as described (3). For luminescence analysis, plants were sprayed with 1 mM D-Luciferin-K-Salt (PJK GmbH) twice within 24 h, and imaged with an Orca 2-BT cooled CCD camera (Hamamatsu Photonics). To quantify the bioluminescence intensity (Fig. S4*B*) of apices versus leaves in mutant plants, 10 plants for each genotype were imaged as described. To avoid saturated spots, unified settings of exposure, gain, and contrast were determined and applied to all images. Luminescence intensity was measured in unprocessed gray-scale pictures using ImageJ. Values were normalized by the measured area size.

RNA Analysis. Total RNA was extracted using TRIZOL reagent (Life Technologies). Reverse transcription was performed on 1–10 μg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative RT-PCR on mature miRNAs, miRNA precursors, and miRNA targets was executed with biological duplicates and technical triplicates, using *BETA-TUBULIN2* (At5g62690) or *ACTIN2/8* (At3g18780/ At1g49240) as reference. RNA blots were performed as previously described (3). In situ hybridization was performed as described (36). The probe spans nucleotides 713 and 1959 of the *RCF3* cDNA. Sequences of oligonucleotides used for RT-qPCR experiments and RNA blots can be found in Table S2.

Small RNA Sequencing and Analysis. RNA for two biological replicates was extracted using TRIZOL reagent for each genotype. Sequencing libraries were prepared with the NEBNext (Set 1) and the TruSeq Small RNA Library Prep Set for Illumina (V2) kit. Raw 50-bp reads were sorted by barcode and adapter and quality trimmed using SHORE v0.9.0 (37). Only reads with a 3′ adapter sequence and trimmed size of 17–25 nt were aligned with bwa v0.7.12 and zero mismatches to the TAIR10 genome (Athaliana_167; phytozome.jgi.doe.gov/pz/portal.html), or the *A. thaliana* miRBase hairpin and mature miRNA sequences (miRBase.org, release 21).

Perfectly matched reads for each miRNA and miRNA* were counted and normalized to the total number of mapped reads per library. Small RNA hierarchical clustering, at each miRNA locus, was performed by normalized coverage of 18- to 24-nt-long small RNA reads in windows extending 20 bp on both sides of the mature miRNA sequence. The sRNA-seq datasets were deposited in the European Nucleotide Archive (ENA) under accession number PRJEB10589.

Protein Analyses. For subcellular localization and bimolecular fluorescence complementation (BiFC) assays, *N. benthamiana* leaves were harvested 3 d after transient transformation and imaged with a TCS SP2 (Leica) or an FV1000 (Olympus) confocal microscope. For BiFC, a nonrelated nuclear protein coding sequence (AT2G29210) was cloned into the corresponding vectors and used as an empty vector (EV) counterpart. Detection of phosphoisoforms was done as previously described (3). Yeast two-hybrid assays were performed using the ProQuest Two-Hybrid System (Life Technologies). For reduction of CPL1 autoactivation, the selection medium was supplemented with 25–150 mM 3-amino-1,2,4-triazole (3-AT).

Note. During the revision of this article, a second report independently confirmed our observation of RCF3 acting in the miRNA biogenesis, reporting aberrant overaccumulation of miRNA*s in 12-d-old *rcf3* seedlings (26).

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Supporting Information

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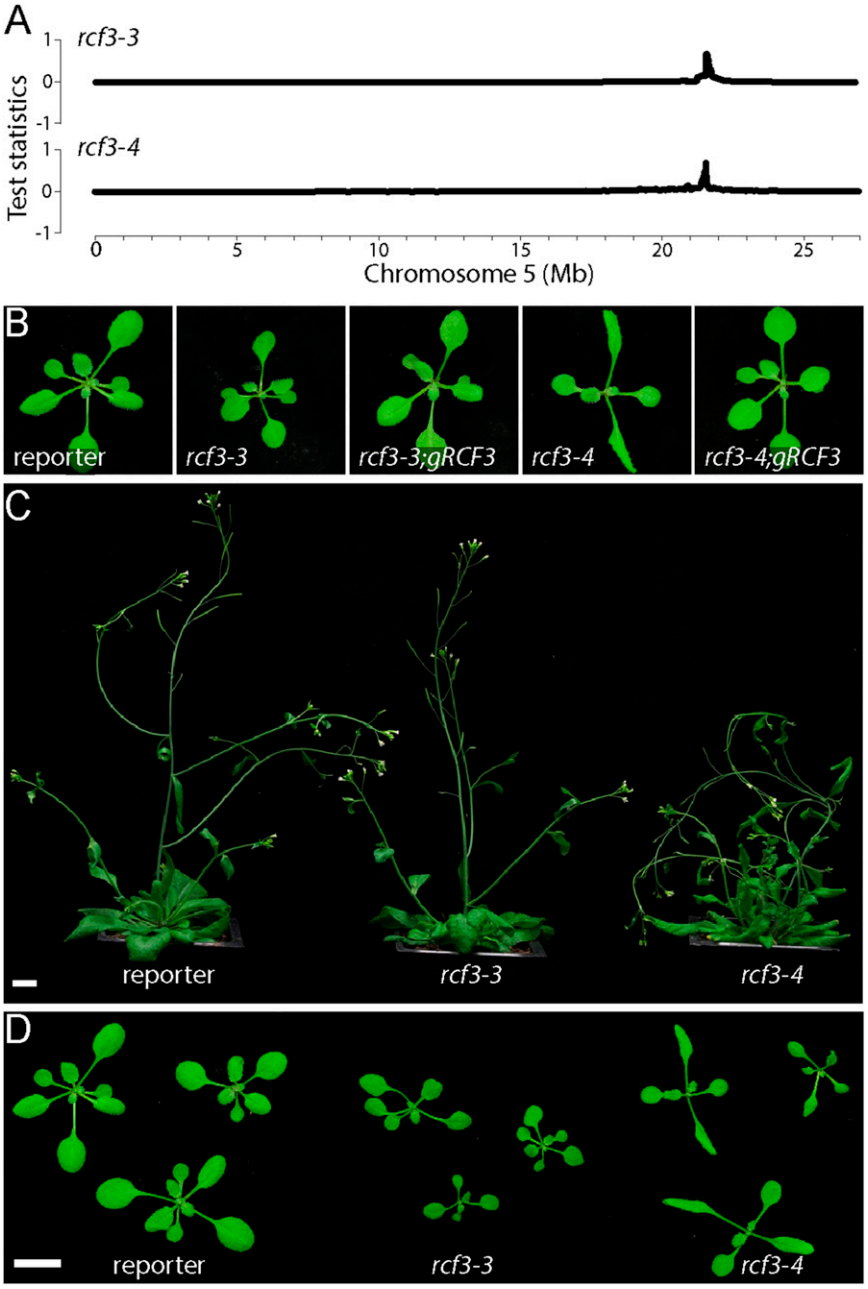

Fig. S1. Phenotype of *rcf3* mutant and rescued plants. (*A*) Chromosome 5 SHOREmap results for *rcf3-3* and *rcf3-4* mutants. (*B*) The 14-days-old *rcf3* mutants with and without a genomic *RCF3* rescue construct. (*C and D*) The 32- and 14-days-old *rcf3* mutant plants. In both panels, plants were imaged individually and mounted in a single black background square to facilitate the comparison and observation. (Scale bars: 1 cm.)

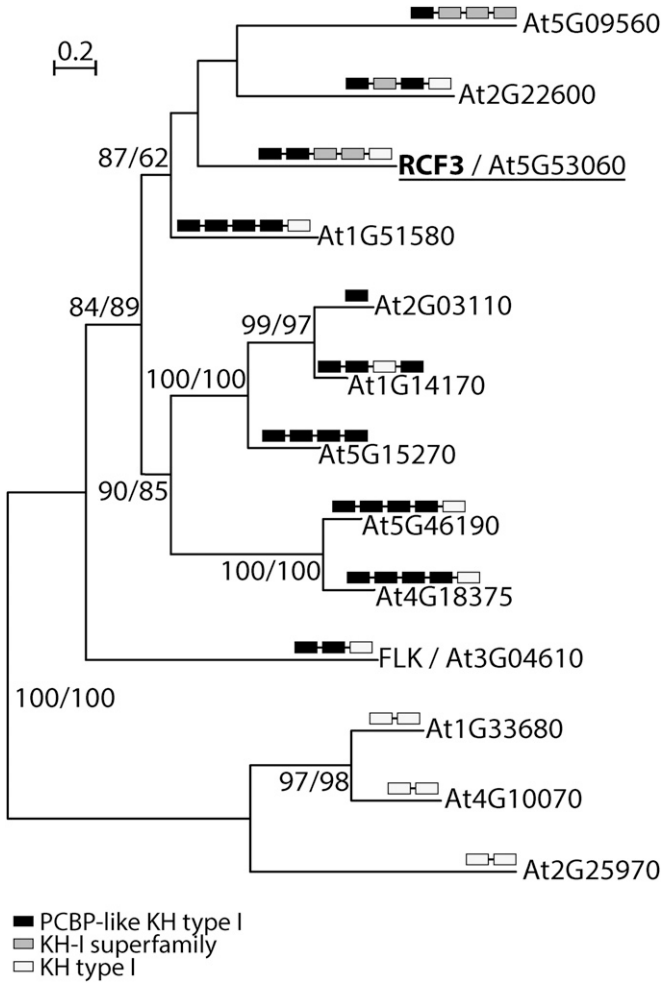

Fig. S2. Phylogenetic analysis of RCF3 homologs. RCF3-like KH domain proteins from *A. thaliana*. Approximate likelihood fraction (aLRT) and bootstrap values (BT) larger than 50% are shown at nodes. Black, gray, and white boxes indicate types of KH domains. The full-length RCF3 protein sequence was compared by BLASTP against the *A. thaliana* RefSeq protein database. All hits were analyzed for the identity of their annotated functional domains using both the National Center for Biotechnology Information (NCBI) conserved domain search engine (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and Pfam version 27.0 (pfam.xfam.org/search/sequence). With MEGA version 5, complete protein sequences were aligned using Muscle, and a phylogenetic tree was estimated with the Maximum Likelihood method. FASTA-formatted alignments were loaded into SeaView Version 4.5.3 for computation of trees, using the best fitted model (WAG) including the approximate likelihood fraction (aLRT) and bootstrapping (100×).

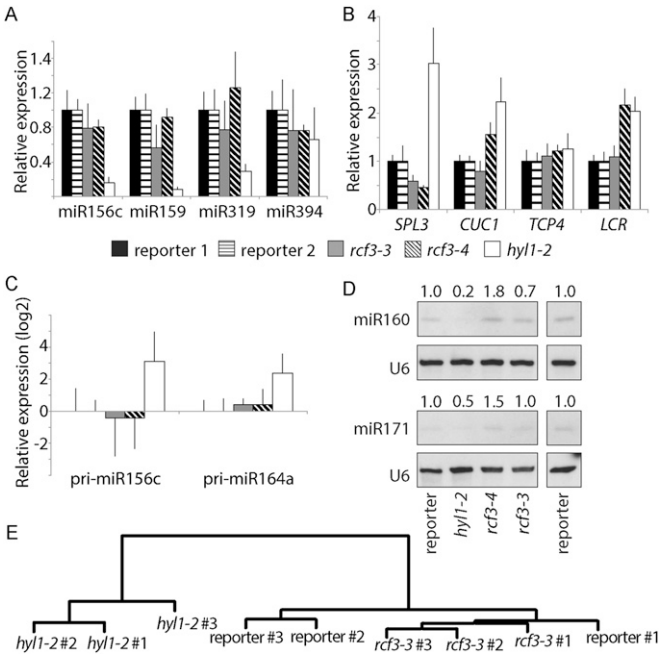

Fig. S3. Effects of *rcf3* mutation on miRNA accumulation and action in fully expanded leaves. (*A–C*) Expression of mature miRNAs, miRNA targets, and miRNA precursors as measured by RT-qPCR. Error bars indicate 2× SEM. (*D*) RNA blots for detection of mature miR160 and miR171, with U6 as loading control. Signal intensity was calculated with ImageJ and normalized to U6. Above each gel, the ratio of signal intensities of mutants to reporter control is noted. (*E*) Hierarchical clustering of normalized genome-wide small RNA coverage profiles within miRNA regions extending 20 bases either side of all mature miRNAs.

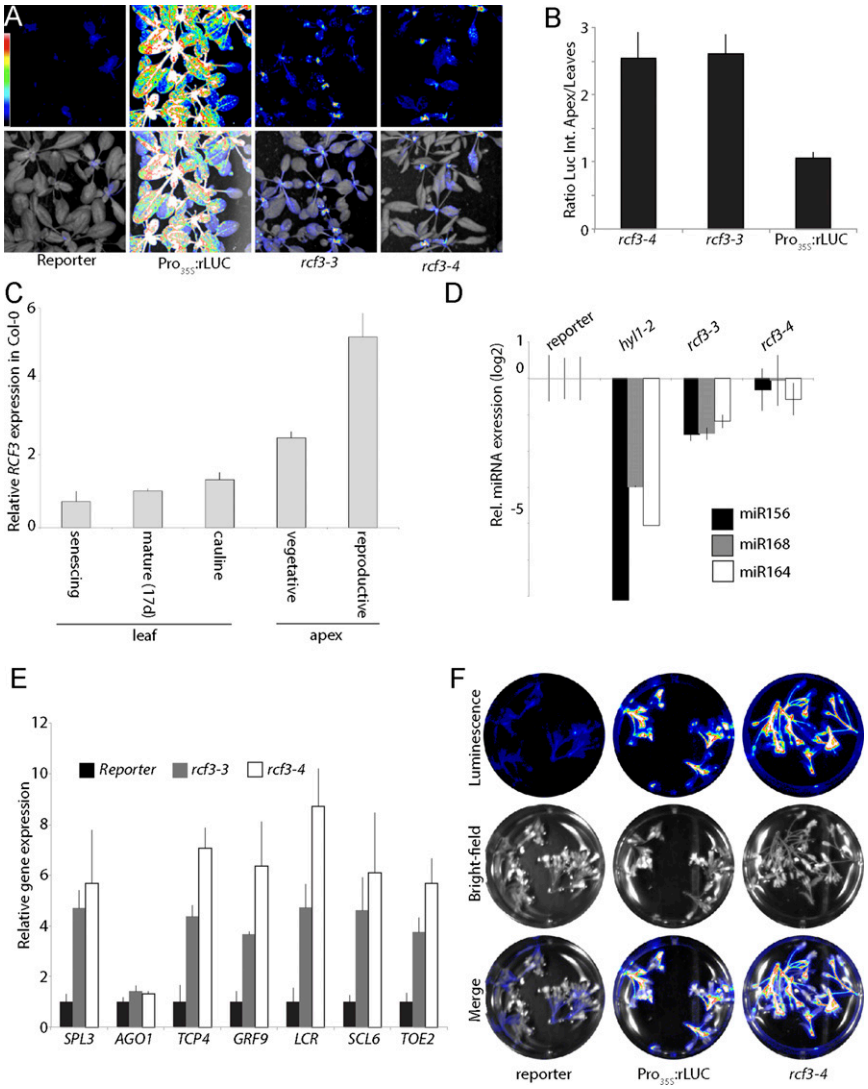

Fig. S4. *RCF3* expression and activity. (*A*) Bioluminescence activity in 17-d-old mutant plants, indicating preferential restoration of reporter activity in younger tissue around the shoot apex. (*Top*) Luminescence. (*Bottom*) Luminescence merged with bright field image. Colored scale indicates low (blue) to high (white) luminescence. (*B*) Ratio of luminescence intensity between shoot apex and leaves. Error bars indicate 2× SEM. (*C*) Expression of *RCF3* mRNA as measured by RT-qPCR. Error bars indicate 2× SEM. (*D* and *E*) Expression of mature miRNAs and miRNA targets as measured by RT-qPCR in inflorescences. Error bars indicate 2× SEM. (*F*) Bioluminescence activity in inflorescences, indicating restoration of reporter activity in reproductive tissues. (*Top*) Luminescence. (*Middle*) Bright field image. (*Bottom*) Merge. Colored scale indicates low (blue) to high (white) luminescence.

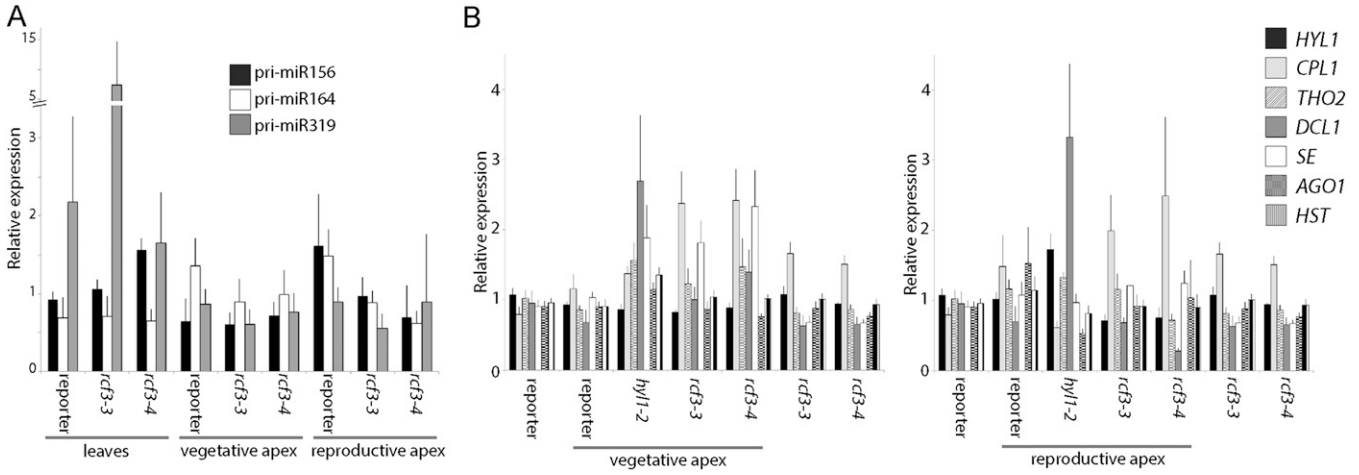

Fig. S5. Expression levels of pri-miRNAs (*A*) and miRNA processing factors (*B*) as measured by qRT-PCR in leaves and vegetative and reproductive apices. Error bars indicate 2× SEM.

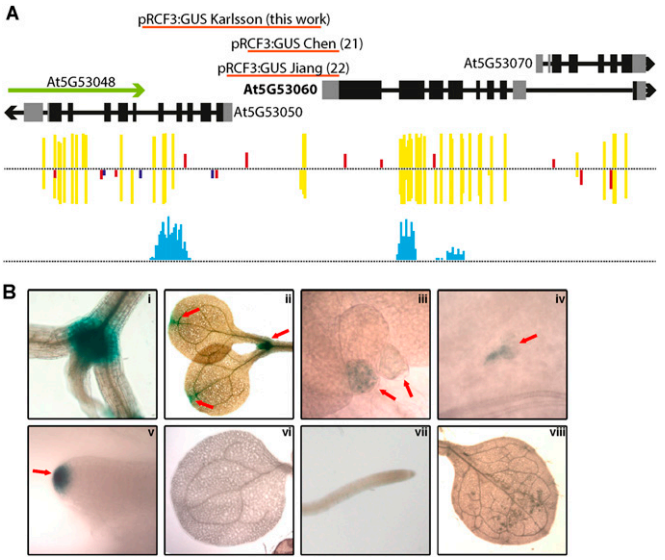

Fig. S6. RCF3 genomic location. (*A*) Schematic representation of the RCF3 genomic neighborhood. Red lines represent the promoter fragments used in this work and in refs. 21 and 22. In yellow, red, and blue, CG, CHH, and CHG methylcytosine marks are noted as detected by ref. 38. Light blue indicates the region where small RNAs mapped. (*B*) Histochemical detection of the Pro_{RCF3}:GUS reporter activity. (*i* and *ii*) Specific GUS activity detected in T1 transgenic plants. (*iii–v*) Reduced and localized activity in transgenic T2 apices. (*vi–viii*) No detectable GUS activity in in T2 cotyledons, root tip, and leaves.

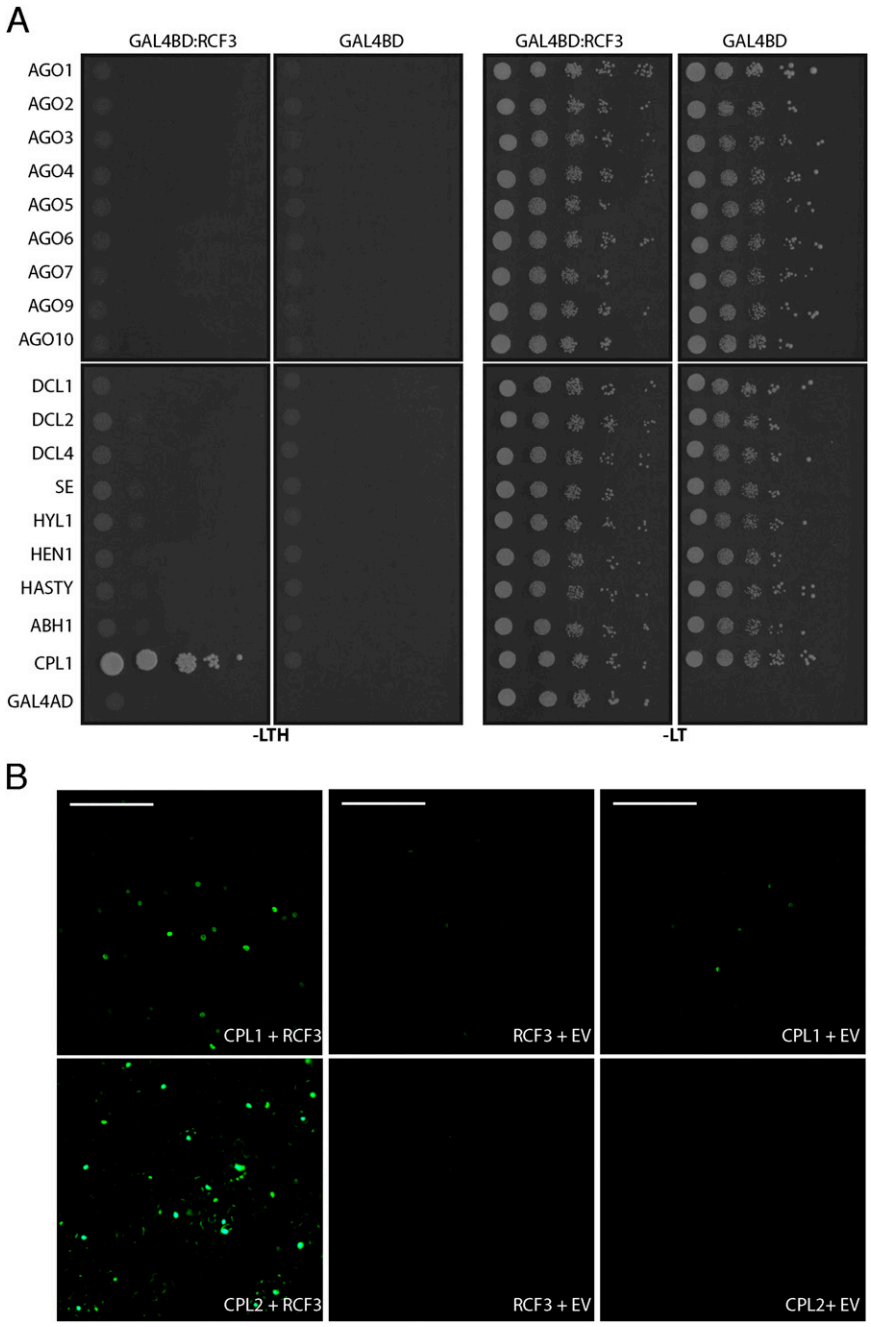

Fig. S7. RCF3 Y2H interaction screen with miRNA factors. (*A*) Yeast transformants were plated on selective medium without leucine, tryptophan (–LT), and histidine (–LTH). All miRNA-related factors were cloned fused to the GAL4 activation domain (AD) whereas RCF3 to the GAL4 binding domain (BD). Serial dilutions of the yeast were plated to ensure even initial yeast density. (*B*) BiFC assay in *N. benthamiana* cells. Empty vector (EV) cotransformation was used as negative control. (Scale bars: 0.2 mm.)

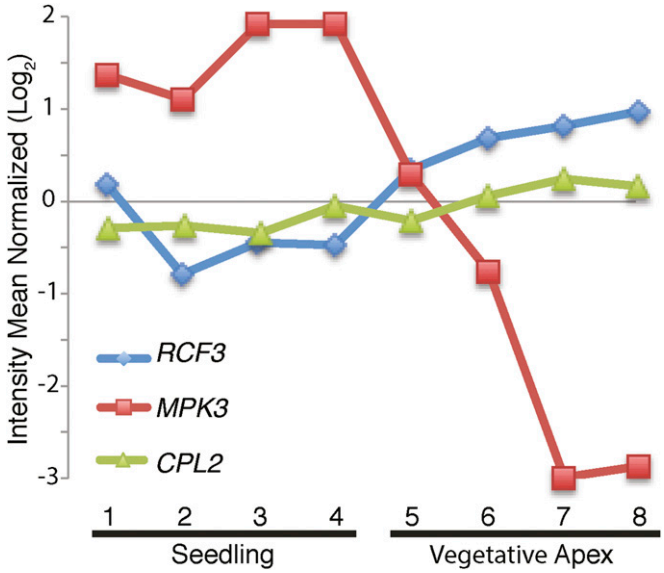

Fig. S8. *RCF3*, *CPL2*, and *MPK3* expression profiles. mRNA abundance, quantified by Affymetrix microarrays, was extracted from the AtGenExpress database (25) and expressed as the mean of the normalized reads. Points 1 and 2, 8-d-old seedling; 3 and 4, 21-d-old seedling; 5, 7-d-old apex + young leaves; 6, 7-d-old apices; 7, 14-d-old apices before bolting; 8, 21-d-old apices after bolting.

Table S1. Plasmids

Name	ID	Description
35S:rLuc	pPM118	Pro_{35S} driving artificial miRNA targeting luciferase expressed from the same vector. The luciferase cDNA was mutated to escape the amiRNA silencing.
miRNA-activity reporter	pPM085	Pro_{35S} driving artificial miRNA targeting luciferase expressed from the same vector
gRCF3	pFRK007	Genomic RCF3
cRCF3	pPM347	RCF3 cDNA
cRCF3 w/o stop	pPM348	RCF3 cDNA without stop codon
RCF3:eGFP	pPM350	Genomic RCF3 without stop fusion to C-terminal citrine
DCL1:mCherry	pPM115	Pro_{35S} driving mutated Cherry (mCherry) N-terminal-fusion to DCL1
SE:mCherry	pPM050	Pro_{35S} driving mutated Cherry (mCherry) N-terminal-fusion to SE
CPL1:mCherry	pPL015	Pro_{35S} driving mutated Cherry (mCherry) N-terminal-fusion to CPL1
GAL4AD:RCF3	pPM384	GAL4 activation domain fusion to RCF3
GAL4BD:RCF3	pPM385	GAL4 binding domain fusion to RCF3
GAL4AD:CPL1	pPM358	GAL4 activation domain fusion to CPL1
GAL4BD:CPL1	pPM359	GAL4 binding domain fusion to CPL1
GAL4AD:DCL1	pPM258	GAL4 activation domain fusion to DCL1
GAL4AD:DCL4	pPM261	GAL4 activation domain fusion to DCL4
GAL4AD:SE3	pPM275	GAL4 activation domain fusion to SE3
GAL4AD:HYL1	pPM273	GAL4 activation domain fusion to HYL1
GAL4AD:HEN1	pPM271	GAL4 activation domain fusion to HEN1
GAL4AD:HASTY	pPM272	GAL4 activation domain fusion to HASTY
GAL4AD:ABH1	pPM274	GAL4 activation domain fusion to ABH1
GAL4AD:CPL2	pPL004	GAL4 activation domain fusion to CPL2
GAL4AD:AGO1	pPM262	GAL4 activation domain fusion to AGO1
GAL4AD:AGO7	pPM268	GAL4 activation domain fusion to AGO7
GAL4AD:AGO9	pPM269	GAL4 activation domain fusion to AGO9
GAL4AD:AGO10	pPM270	GAL4 activation domain fusion to AGO10
GAL4BD:CPL2	pPL003	GAL4 binding domain fusion to CPL2
Pro35S:mHYL1	pPM444	Pro_{35S} driving mutated HYL1 phosphomimic Ser > Asp (last Ser > Glu)
Pro35S:mHYL1	pPM445	Pro_{35S} driving mutated HYL1 phosphomimic Ser > Ala
cRCF3	pPL036	RCF3 (713–1959) fragment in pGEM
Pro35S:CPL2:N Citridine	pPL022	BiFC CPL2 construct
Pro35S:CPL2:C Citridine	pPL023	BiFC CPL2 construct
Pro35S:CPL1:N Citridine	pPM362	BiFC CPL1 construct
Pro35S:RCF3:C Citridine	pPM388	BiFC RCF3 construct
Pro35S:RCF3:N Citridine	pPM389	BiFC RCF3 construct
Pro35S: AT2G29210:C Citridine	pPM375	BiFC "Empty Vector" construct
Pro35S: AT2G29210:N Citridine	pPM376	BiFC "Empty Vector" construct
ProRCF3:GUS	pPM378	RCF3 promoter:GUS fusion

Constructs for plant transformation are based on pGREEN and confer either Basta or kanamycin resistance in plants.

Table S2. DNA oligonucleotide primers and probes

Gene	Sequence	Purpose
RCF3 genomics	F:GGAGGTTAGGACTGCCACGTA	Cloning
	R:GTACAAGAGGATGGACCGTGA	
RCF3 cDNA	F:ATGGAGAGATCTAGATCCAAGAG	Cloning
	R:GAGCATACAAGAGGATGGACCGTGA	
RCF3 cDNA	F:TCGGATTCTTCCAAGAGAAAG	Splicing detection
	R:GACGAGATGATACAATGGCTAAA	
RCF3 cDNA w/o stop	R:GAGCATACAAGAGGATGGACCG	Cloning
CPL2 cDNA	F:ATGAATCGTTTGGGTCATAAAT	Cloning
	R:TATGAAACCTTGCACCCAAGGCT	
miR160	TGGCATACAGGGAGCCAGGCA	RNA blot
miR171	GATATTGGCGCGGCTCAATC	RNA blot
U6	GCTAATCTTCTCTGTATCGTTCC	RNA blot
miR156c	F:GCGGCGGTGACAGAAGAGAGT	qRT-PCR
miR159	F:GCGGCGTTTGGATTGAAGGGA	qRT-PCR
miR319	F:CGTCGTTGGACTGAAGGGAG	qRT-PCR
miR394	F:CGCCATGTTGGCATTCTGTCC	qRT-PCR
miRNA RT	R:GTGCAGGGTCCGAGGT	qRT-PCR
pri-miR156c	F:ACTCCAACACCTTCAAAGTCTGC	qRT-PCR
	R:GAGAGAGAAAGTGAGAGATGGGAAC	
pri-miR164a	F:CCCTCATGTGCTTGGAAATG	qRT-PCR
	R:GCAAATGAGACGGATTTCGTG	
SPL3	F:ACGCTTAGCTGGACACAACGAGAGAAG	qRT-PCR
	R:TGGAGAAACAGACAGAGACACAGAGGA	
CUC1	F:GAAGAGTTGTTGGGTCATGC	qRT-PCR
	R:CGAAATCAATCTGTCCCGATG	
TCP4	F:CAACCGATACAGGAAACGGAG	qRT-PCR
	R:CTGGTATGCGAAAACCCGAAG	
LCR	F:CTATCCACAGCACACTTTAC	qRT-PCR
	R:CACAGCATTGTAGGTTATCAG	
BETA-TUBULIN2	F:GAGCCTTACAACGCTACTCTGTCTGTC	qRT-PCR
	R:ACACCAGACATAGTAGCAGAAATCAAG	
RCF3	F:CCTAAGCTCGTTACGAAATCAAGA	qRT-PCR
	R:TCACGGTCCATCCTCTTGTATGCTC	
RCF3	F:TGAAAAACGCTTTAGCCATTG	In situ probe

Dataset S1. Small RNA sequencing results

Dataset S1

Normalized counts of the miRNA and miRNA* in control, *hyl1-2*, *rcf3-3*, and *rcf3-4* vegetative apices.

III

A role for the F-box protein HAWAIIAN SKIRT in plant miRNA function

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INTRODUCTION

MicroRNAs (miRNAs) are 21 to 24 nucleotide (nt) long single-stranded RNA molecules that are crucial for regulation and fine-tuning of gene expression in multicellular organisms (Bartel 2009). In plants, transcription of *MIR* genes generates longer precursor RNAs with characteristic stem-loop folding, and is followed by two major nuclear processing steps mediated mainly by the endoribonuclease DICER-LIKE1 (DCL1), and aided by cofactors including SERRATE (SE), HYPONASTIC LEAVES1 (HYL1) (Kurihara and Watanabe 2004; Laubinger et al. 2008; Vazquez et al. 2004; Bernstein et al. 2001). The first dicing step, usually near the base of the precursor miRNA stem, excises the primary miRNA (pri-miRNA) stem-loop, which is cut once more to remove the loop and form the miRNA/miRNA* duplex. After 3'-O methylation and translocation to the cytoplasm (Yu et al. 2005), the mature miRNA (guide) strand forms, through association with an ARGONAUTE protein, an active miRNA INDUCED SILENCING COMPLEX (miRISC). By sequence complementarity of the miRNA to its mRNA targets, miRISC selectively imposes inhibition of translation or transcript cleavage (Rogers and Chen 2013; Brodersen et al. 2012).

Transcriptional regulation assures the correct spatio-temporal expression of *MIR* genes, and post-transcriptional steps can further fine-tune miRNA accumulation. Additionally, mature miRISC activity can be attenuated by miRNA target mimicry (Franco-Zorrilla et al. 2007), where an RNA containing a sequence motif that is complementary to a miRNA or miRNA family sequesters the respective miRISC, which is then no longer available for inhibition of regular mRNA targets (Franco-Zorrilla et al. 2007). Only a single case of natural miRNA target mimicry, of *IPS1*, which interferes with the activity of miR399, has been studied in detail in plants (Franco-Zorrilla et al. 2007). In animals, this principle is known as competing endogenous RNAs (ceRNAs) and is thought to be rather widespread (P. Wang et al. 2015; Bak and Mikkelsen 2014).

Many conserved plant miRNAs are encoded by medium-size gene families (A. Li and Mao 2007). This has created challenges for the study of their biological function through genetic approaches, a limitation overcome by the generation of a collection of artificial miRNA target mimicry lines (MIMs) to knock down the majority of *Arabidopsis thaliana* miRNA families one by one (Todesco et al. 2010). Artificial mMiRNA target mimicry can be based on the endogenous *IPS1* transcript, or on

entirely artificial sequences (Franco-Zorrilla et al. 2007; Todesco et al. 2010; Yan et al. 2012; Reichel et al. 2015).

The deeply conserved miR156 miRNA family is encoded by eight *MIR* genes in *A. thaliana*, miR156a to miR156h (Griffiths- Jones 2004; Kozomara and Griffiths-Jones 2011; Griffiths-Jones et al. 2008; Griffiths-Jones 2006; Griffiths-Jones et al. 2006; Kozomara and Griffiths-Jones 2014). Mature miR156 strongly accumulates in shoots early in the life of the plant and gradually decreases in abundance as the plant ages (J.-W. Wang 2014; J.-W. Wang, Czech, and Weigel 2009; Wu et al. 2009; Wu and Poethig 2006). It negatively regulates the abundance of at least 10 out of 16 members of the SQUAMOSA PROMOTER-BINDING (SPB) PROTEIN-LIKE (SPL) transcription factor family, which control a range of biological processes, most of them relating to developmental progression during the vegetative phase of plant growth (Xu et al. 2016).

Repression or loss of miR156 function as it can be observed in plants expressing the MIM156 construct, or plants expressing miR156-insensitive versions of the *SPL* targets, induces adult growth traits, such as serrated leaf margins, prematurely (J.-W. Wang et al. 2008; Wu and Poethig 2006; Franco-Zorrilla et al. 2007). Also the rate at which rosette leaves are initiated during vegetative growth is greatly reduced, and MIM156 cotyledons are bent and spoon-shaped (Todesco et al. 2010). Contrastingly, ectopic overexpression of miR156, which reduces SPL levels, prolongs the juvenile phase with non-serrated rosette leaves, and plants initiate rosette leaves faster than wild-type (Wu and Poethig 2006).

Here, we identify mutations in the *HAWAIIAN SKIRT* (*HWS*) F-box gene (At3G61590) as suppressors of MIM156 and other miRNA target mimicry phenotypes. As part of an Skp-Cullin-F-box (SCF) complex, F-box proteins give E3 ubiquitin ligases target specificity via recognition of substrates for degradation by the 26S proteasome (Risseeuw et al. 2003). HWS was previously shown to interact with the classical components of an SCF complex, ASK20A and ASK20B, the two translational products of *ARABIDOPSIS SKP1-like 20* (*ASK20*), and with ARABIDOPSIS SKP-LIKE1 (ASK1), in a Y2H screen, and ASK1 functioned as bridge between CULLIN1 (CUL1) and HWS (Ogura et al. 2008; Kuroda et al. 2002). Due to delayed abscission and sepal fusion, *hws* mutants fail to shed sepals, petals and anthers (Gonzalez-Carranza et al. 2007). Loss of *HWS* furthermore results in increased organ growth, whereas overexpression yields smaller plants with

elongated, serrated and hyponastic leaves, thus reminding of phenotypes previously described for the miRNA biogenesis factor mutants *hyl1-2* as well as hypomorphic *ago1 (Gonzalez-Carranza et al. 2007)*.

We found that *HWS* also plays a role in plant miRNA function. Mutations in *HWS* suppress the characteristic developmental defects not only of MIM156 expressing plants, but also of several additional MIM lines and increase the steady-state levels of miRNAs, a phenotype mirrored by decreased miRNA target abundance. Overexpression of full-length HWS has an inverse effect on the levels of miRNAs and their targets, and this function requires its F-box domain. The characteristic delayed floral organ abscission ('skirt') phenotype of *hws* mutants is lost when combined with mutants of several miRNA biogenesis players, indicating that *HWS* and miRNA factors like *SE*, *HYL1* and *AGO1* are epistatic to each other and active in a common pathway. We propose that HWS is a new factor involved in the biogenesis of miRNAs, and exerts its function through an F-box mediated molecular process.

RESULTS

hawaiian skirt mutations suppress miRNA target mimicry induced phenotypes

To identify genetic modifiers counteracting the activity of a MIM156 transgene, we focused on three easily monitored developmental abnormalities characteristic for lines ectopically expressing the artificial transgene: spoon-shaped cotyledons, premature rosette leaf serration, and a reduced leaf initiation rate during vegetative growth. In one line that we identified from an M2 pool of EMS-mutagenized MIM156 seeds, all investigated phenotypic alterations were suppressed. We localized the causal mutation to a region on the right arm of chromosome 3 using mapping by sequencing (Figure 1A and B; see Materials and Methods for details).

Within this region, a G to A single nucleotide substitution (G537A, chr3:22793585, TAIR10) was identified that caused a premature termination codon (W179STOP) in *HAWAIIAN SKIRT* (*HWS*; At3g61590). HWS is a 412 amino acid

(aa) protein with an N-terminal F-box domain, a putative trans-membrane domain, and a C-terminal Kelch-2 domain (Figure 1C). To confirm that this mutation was responsible for the phenotypic suppression of the characteristic MIM phenotypes, we tested for complementation by transforming the isolated mutant with a genomic construct of *HWS*. The MIM156 phenotype was restored, confirming that *HWS* was indeed the causal locus (Figure 1A). We henceforth refer to the mutant allele as *hws-3*. RT-qPCR detecting the *HWS* transcript shows similarly decreased levels in *hws-3* as in *hws-1* plants (see below), suggesting that *hws-3* is a hypomorphic allele (Figure S1F).

Two other *hws* mutant alleles have previously been isolated and shown to be impaired in the abscission of floral organs, *hws-1* and *hws-2* (Gonzalez-Carranza et al. 2007). Incomplete separation of sepals imposes a structural barrier that prevents the shedding of sepals, petals and stamens (Gonzalez-Carranza et al. 2007). Both with and without the MIM156 transgene, we observed similarly impaired abscission in *hws-3* plants (Figure 1D, Supp. Figure S1A, B and C). Partial fusion of cauline leaves to the inflorescence stem was evident in both *hws-1* and *hws-3* mutants, and like sepal abscission persisted in the presence of the MIM156 transgene (Figure S1E).

To identify a molecular role of HWS that could explain the suppression of MIM156 induced defects, we first tested if other mimicry lines were equally affected. We combined *hws-1* with three additional mimicry transgenes - MIM159, MIM164 and MIM319 - each of which shows distinct developmental alterations, including hyponastic leaves in MIM159, increased leaf serrations in MIM164, and reduced fertility in MIM319 lines (Todesco et al. 2010). We found that the *hws-1* mutation could suppress the characteristic phenotypes of all three mimicry lines (Figure 2A), pointing to a role of HWS upstream of MIM156-specific factors like the miR156-targeted *SPL* transcripts. As expected, presence of the MIM319 and MIM159 transgenes did not affect the *hws* abscission phenotype (Figure S2A and see below).

Further support for a more general role of HWS came from introducing a miR156 resistant SPL9 (rSPL9) transgene into *hws* mutants. This transgene expresses a version of SPL9 that avoids regulation by miR156 due to the presence of five base substitutions in its miR156 target site (J.-W. Wang et al. 2008). Like MIM156, rSPL9 plants accumulate higher levels of SPL9 and thus display MIM156-

like phenotypes including spoon-shaped cotyledons and a slower leaf initiation rate (Figure 2D,). We found that the rSPL9 phenotype was similar in wild-type and *hws-3* backgrounds (Figure 2D), pointing to a role for HWS upstream of miRNA target stability and/or activity, consistent with its generic ability to suppress MIM phenotypes.

A more general role of HWS could impede MIM action directly at the level of the (endogenous or modified) *IPS1*-based MIM transcript, or further upstream, affecting miRNA accumulation. Endogenous *IPS1* expression is naturally low but greatly induced upon Pi starvation (Martín et al. 2000). To monitor potential alterations of endogenous *IPS1* accumulation when *HWS* function is impaired, we measured its steady-state levels both under normal and Pi starvation conditions (see Materials and Methods). Using RT-qPCR, we saw that *IPS1* accumulation is still strongly increased in *hws*-3 mutants grown in Pi-starvation medium; levels are even higher than in the wild type, both at high and low Pi supply (Figure 2B). One would expect a more efficient sequestration of miRNAs when more mimicry transcripts (i.e. *IPS1*) are available, and consequently a release of miRNA-mediated target suppression, from this observation. We observed the opposite, as *PHO2*, the endogenous target of *IPS1*-bound miR399, was less rather than more up-regulated in Pi-limiting conditions compared to the control. Similarly, genetic suppression of MIM phenotypes by *HWS* mutations predicts reduction rather than over-accumulation of miRNA targets in *hws* mutants, although feedback loops in the *IPS1-miR399-PHO2* module (Franco-Zorrilla et al. 2007; Fujii et al. 2005; Bari et al. 2006; Chiou et al. 2006) might complicate our interpretations.

The suspicion of feedback regulation confounding our observations was reinforced when we turned to plants harboring a MIM transgene: *IPS1*-based engineered transcripts, now uncoupled from promoter-based feedback loops, were reduced in *hws-3* mutants (Figure 2C). In concordance, the corresponding miRNAs over-, and their targets under-accumulate, an effect that was seen for both of the examined lines, MIM156 and MIM164 (Figure 2C), and was also consistent with reduced *PHO2* levels in *hws-3* plants grown in Pi limiting media (Figure 2B). This observation suggests that HWS might somehow stabilize mimicry transcripts, yet this effect could also be an indirect consequence of altered miRNA levels further upstream in the pathway.

HWS suppresses miRNA accumulation

The previous observations indicated that HWS could be involved more broadly in miRNA regulation. This was further supported by a general resemblance of phenotypic defects in *hws* mutants and those observed in mutants with defects in the miRNA biogenesis pathway, or those with altered levels of miR164, a miRNA unrelated to our genetic screen: *hws* mutants show reduced serrations (Figure S3A) - a trait that is often affected in miRNA biogenesis mutants (Laubinger et al. 2008; Morel et al. 2002). Moreover, both the 'skirt', and cauline leaf fusions to the stem have been described as results of miR164 overexpression (Schwab et al. 2005; Mallory, Reinhart, et al. 2004). Further, when we overexpressed the *HWS* coding sequence from the constitutive *35S*::CaMV promoter, similar to what was described earlier, plants developed severe abnormalities, including upwards-pointing, highly serrated and hyponastic leaves (Figure 3A) (Gonzalez-Carranza et al. 2007). This was also reminiscent of mutants with impaired miRNA activity, for example *hyl1-2*, *ago1-25* and *ago1-27* or *hst-3* (Figure S3C) (Morel et al. 2002; Vazquez et al. 2004; Bollman et al. 2003). We therefore decided to test whether miRNA activity was directly affected in *HWS* overexpressors as well as *hws* mutants. We observed that while miRNA levels tended to be slightly up-regulated in *hws* mutants, they were generally downregulated in *35S::HWS* compared to the wild-type control (Figure 3B). Only miR173 abundance appeared to be largely unaffected, while the effect on miR164 was particularly pronounced. Mirroring this, we also saw a decrease in the levels of miRNA-targeted transcripts of *AGO1* (miR168), *SPL3* (miR156) and *TCP4* (miR319) in *hws* mutants, and a matching increase in *35S::HWS* (Figure 3C). In addition, *IPS1* accumulation in *35S::HWS* was similar to what was seen in wild type, whereas *PHO2*, the miR399 target, was upregulated, independent of Pi supply (Figure 2B). We thus hypothesized that HWS plays a more general role in the miRNA pathway.

The particularly pronounced effects on miR164 and miR156 might be attributed to *HWS* expression being specifically elevated in regions of miR164 and miR156 expression. Both miR156 and miR164 accumulate to high levels in emerging leaves (Nikovics et al. 2006a; Bazzini et al. 2009; J.-W. Wang, Czech, and Weigel 2009; J.-W. Wang et al. 2008; Wu and Poethig 2006), with miR164 also showing a more restricted expression pattern around the veins and the points of leaf serration

(Figure S3D, (Nikovics et al. 2006a)). A similar expression pattern was seen in Pro_{HWS}::GUS plants (Figure S3D). This is in agreement with the reduction of serration found in *hws* plants, as in the emerging leaves of wild-type plants, miR164 acts as a suppressor of leaf serration by targeting members of the *CUC* family of transcription factors (Figure S3A, (Nikovics et al. 2006b).

Interaction of HWS with general miRNA factors

To further substantiate a connection between *HWS* and miRNA biogenesis factors, we tested for genetic interactions. For this purpose, we prepared crosses of *hws-1* with mutant alleles of *AGO1* (*ago1-25*, *ago1-27*; (Morel et al. 2002)), *ABH1* (*abh1-753*; (Laubinger et al. 2008)), *HST* (*hst-3*; (Bollman et al. 2003)), *HYL1* (*hyl1-2*; (Vazquez et al. 2004)), and *SE* (*se-3*; (Laubinger et al. 2008)) (Figure 4). In all cases, double-homozygous F_2 plants resembled the single mutants in the miRNA biogenesis pathway (Figure 4A) - and the *hws* 'skirt' phenotype was reduced (Figure 4B). This indicates that the miRNA factors are epistatic to and share a common pathway with HWS.

Owing to its F-box domain, HWS could be acting in an SCF-complex, conferring specificity for an unknown substrate. Previously shown HWS interaction with the common SCF component *Arabidopsis*-Skp protein ASK1 (Ogura et al. 2008; Kuroda et al. 2002) and involvement of F-box proteins like the viral suppressor P0 (Pazhouhandeh et al. 2006; Bortolamiol et al. 2007; Baumberger et al. 2007) and F-BOX WITH WD-40 2 (FBW2, (Earley et al. 2010)) with the miRNA context substantiates the idea that HWS might impact miRNA function through destabilizing a protein directly involved in miRNA biogenesis or processing. Only full-length *35S::HWS* complemented the *hws-1* phenotype, whereas transformants expressing a version that lacks the F-box domain (*35S:mHWS* transgene) retained the *hws*-characteristic fused sepals and cauline leaves (Figure S1 A, B, E). Furthermore, 35S::mHWS did not induce the *hyl1-2* or *ago1*-like phenotypes observed in 35S::HWS plants (Fig 3A, 5A). Consistently, steady-state levels of miRNAs and their targets in 35S::mHWS were closer to wild type (Figure S3E, F).

To unbiasedly detect HWS-interactors *in planta*, we harvested rosette leaves to immunoprecipitate the GFP-fused 35S::HWS with a GFP-antibody and performed

mass spectrometry (MS). As controls, we processed both 35S::mHWS as well as 35S::GFP-expressing tissue to test for F-box specificity of the interactions. Enrichment of ASK1 and two other SCF-complex proteins, ARABIDOPSIS SKP-LIKE 2 (At5g42190) as well as of CULLIN1 (At4g02570), in the 35S::HWS fraction, but in neither of the two controls, supported HWS function as a classical F-box protein. Beyond this, we could however not observe significant associations of HWS with known miRNA-related proteins. It is thus possible that HWS contributes to the miRNA pathway through a protein not yet described in this context, that an interaction with an already known factor is rather weak and transient as described for other F-box proteins (Earley et al. 2010; Coyaud et al. 2015), or that the concentration of the interactor is very low and therefore not detectable by our approach. Using a Yeast-Two-Hybrid assay (Y2H; (Manavella et al. 2012; Fields and Song 1989)), we thus looked for interaction partners in a more direct way, specifically targeting proteins of the miRNA pathway. We could, however, not detect a physical interaction between the HWS protein and any of the miRNA biogenesis factors tested (Figure S4).

The only miRNA-related protein detected with higher confidence via MS - although present even in the control - was AGO1. It is known to associate with several F-box proteins, among them FBW2, which destabilizes it, and the viral suppressor P0 (Earley et al. 2010; Bortolamiol et al. 2007; Baumberger et al. 2007; Pazhouhandeh et al. 2006; Csorba, Kontra, and Burgyán 2015). Similarities between the HWS overexpression phenotype and *ago1* supported a hypothetical functional connection of the two, and AGO1's role in miRISC assembly fits with a presumed HWS role upstream of miRNA targets. As we did not observe direct interaction of HWS with full-length AGO1 in Y2H assays (Figure S4), we tested a construct comprising only the AGO1 N-domain that was previously successfully used to detect interaction with an F-box protein (P. Brodersen, personal communication). Unfortunately, we did not observe any interaction either (Figure S5A), which was not entirely unexpected, as F-box proteins often recognize their targets only when these are post-translationally modified, and neither P0 and AGO1 interact with each other in yeast (Bortolamiol et al. 2007; Petroski and Deshaies 2005). Co-immunoprecipitation assays *in planta* did however also not detect association of HWS and AGO1 (data not shown).

If the interaction is too transient to be detected, or indirect, mediated by a third, unknown factor, we might still be able to see effects on AGO1 stability, as long as they are not masked by the complex feedback loops balancing AGO1 abundance (Vaucheret et al. 2004; Vaucheret, Mallory, and Bartel 2006). While we observed a positive influence of the presence of its F-box on HWS stability, both in transient expression assays in *N. benthamiana* and stably transformed *Arabidopsis* plants, AGO1 levels were largely unaltered (Figure 5B, S5B). Ubiquitination by a HWS-associated SCF-complex could, apart from targeting for proteasome-mediated degradation, affect AGO1 function in other ways, such as altering cellular targeting that was previously implicated in miRISC function (Mukhopadhyay and Riezman 2007; W. Li and Ye 2008; Brodersen et al. 2012; Gibbings et al. 2009). However, overall as well as AGO1-specific ubiquitination levels appeared unchanged, both in *N. benthamiana* and *Arabidopsis* (Figure S5C).

DISCUSSION

MiRNAs are formed and function through a complex network comprising a multitude of general and highly specialized proteins (Voinnet 2009; Bologna and Voinnet 2014). Using a MIM156-based genetic screen designed to retrieve both mutations with specific effects on the miR156/SPL-pathway, as well as more general effects on miRNA target mimics, or miRNAs in general, we have identified the F-box protein HWS as a new factor involved in miRNA biogenesis, which highlights the usefulness of MIM lines for the identification of negative factors in the miRNA pathway. Overexpression of HWS leads to a decrease in miRNA levels and consequently higher target levels - deprivation of HWS instead, as in *hws-1*, has the inverse effect (Figure 3). The phenotypes of miRNA factor mutants like *ago1-25*, *hyl1-2* and *se-3* are epistatic to the one in *hws-1*. Introduction of *hws-1* into these plants does not change their respective phenotypes, but suppresses the *hws*-typical ‘skirt’ (Figure 4), pointing towards involvement of all factors in a common pathway and thus further substantiating the connection between miRNAs and HWS.

HWS is an F-box protein, and we have shown that the F-box is necessary to execute its function in miRNA biogenesis, yet despite the beautiful genetic

suppression of MIM-induced phenotypes by *hws*, we were not able to unequivocally pinpoint its molecular target(s). Nevertheless, we can conclude several important aspects of HWS's role in the miRNA pathway and in context with MIM transcripts in general:

Expression of MIM transcripts has different effects on the respective miRNAs: some are greatly reduced, whereas others are only mildly affected (Todesco et al. 2010). While this previous observation could be a consequence of the analyzed tissue and cell types, our observation that MIM156 levels are reduced in *hws* mutants (Figure 2C) suggests that additional feedback might take place, affecting the equilibrium between MIM and miRNA accumulation. MIMs largely function like miRNA sponges that have later also been described in the animal field (Ebert, Neilson, and Sharp 2007), specifically sequestering the miRNAs they can bind to. Since these sponges can sequester only a limited amount of miRNAs, overexpression of miRNAs can remedy their effects, a scenario potentially reflected in the miRNA-overexpressing and MIM-suppressing *hws* mutant. Even a small reduction of MIM transcript levels as in MIM164; *hws-1* (Figure 2C) could already be sufficient to tip the equilibrium between MIM and miRNA necessary for MIM function, and cause suppression of the characteristic phenotype.

We can further conclude that the 'skirt' phenotype observed in *hws* mutants is likely caused by overaccumulation of miR164 in floral organs, as skirts are lost in the presence of MIM164 (Figure S2A, S3B). This is consistent with earlier observations showing that continuous overexpression of miR164b, as well as *cuc1 cuc2* double mutants induce fused sepals and stamens and hence floral 'skirts' (Mallory, Dugas, et al. 2004; Nikovics et al. 2006a; Hibara et al. 2006).

As we only observed miRNA-like phenotypes and mutant rescue when overexpressing HWS in full length including its F-box domain (Figure S1A,B,C and 5A), we conclude that we identified another F-box protein involved in the miRNA pathway. HWS was previously shown to possess F-box 'activity' (Takahashi et al. 2004; Kuroda et al. 2002) and confirmed to interact with SCF-complex proteins (Gonzalez-Carranza unpublished, this manuscript, (Arabidopsis Interactome Mapping Consortium 2011)). Other F-box proteins have been implicated in miRNA function before: AGO1, the core protein of miRISC, is targeted for degradation by the viral suppressor and F-box protein P0, and its levels also decrease upon overexpression of the F-box protein FBW2 (Baumberger et al. 2007; Bortolamiol et

al. 2007; Earley et al. 2010; Pazhouhandeh et al. 2006; Csorba et al. 2010). As the only hint from our mass spectrometry and Y2H assays was the enrichment of AGO1 in 35S::HWS-GFP IPs, we performed several biochemical experiments aimed at identifying changes in AGO1 when more or less HWS was present. While this proved unsuccessful, we cannot exclude AGO1 as a true HWS target yet, as previous related experiments by others were also only partially successful: increase of AGO1 levels was only detected in *fbw2* mutants in genetic backgrounds compromised in miRNA biogenesis, not in *fbw2* single mutants (Earley et al. 2010). F-box proteins often interact with their targets only transiently, such that detection of these interactions is not trivial, sometimes impossible, especially with heterologous approaches like Y2H (Earley et al. 2010; Bortolamiol et al. 2007). HWS interaction with miRNA biogenesis factors, if present, is likely much weaker and more transient than the HWS F-box connection with SCF complexes, as we could detect clear enrichment of both SKPs and CUL in the MS analysis.

Since the F-box domain usually provides the interaction interface towards the other SCF complex components, HWS would interact with AGO1 or other targets via the Kelch-domain. Elimination of the F-box should stabilize this interaction (Skaar, Pagan, and Pagano 2013). However, co-infiltrations of AGO1 and HWS, with and without the F-box, did not change AGO1 protein or ubiquitination levels (Figure S5B). Either HWS does not mediate AGO1 degradation, or HWS action requires additional, *Arabidopsis*-specific factors, precluding its function in transient assays. Possibly, a bridging factor is necessary for HWS to indirectly affect miRNA biogenesis, or HWS-mediated targeting depends upon prior target modifications, as for example known for the within F-boxes common phosphorylation-dependent recruitment (Skaar, Pagan, and Pagano 2013).

To identify a specific role for HWS within miRNA biogenesis and function, it will be crucial to find targets of HWS-SCF-complex action, for example through large-scale interaction screen assays or monitoring of protein modification/abundance changes upon HWS overexpression. Since HWS over-accumulated to higher levels without the F-box (Figure 5B), and as 35S::HWS, but not 35S::mHWS plants, display a strong miRNA-related phenotype, it is also possible that HWS is part of a feedback-loop involving miRNAs. Interaction with, recognition of or activity on its target might destabilize HWS, explaining the observed stabilized protein levels in plants lacking the F-box. Which miRNA factors and miRNAs are

potentially involved in such a HWS-associated loop remains to be discovered in further studies.

Apart from proteomics assays, another obvious experiment to pinpoint where HWS enters the miRNA picture is to indirectly capture HWS action by monitoring genome-wide transcript levels via RNA-seq. Differential expression analyses combined with GO term analysis will provide information to narrow down the whereabouts and dimensions of HWS action, and co-expression networks relating to known miRNA factors can further help to narrow down its biochemical point of action.

MATERIAL AND METHODS

Plant Material

Arabidopsis thaliana seeds of the Col-0 accession were surface sterilized with 10% bleach, 0.5% SDS and stratified for 2 to 3 days at 4°C. Plants were grown at 23°C either on Murashige Skoog (MS) plates (1/2 MS, 0.8% agar, pH 5.7) or in soil in either short day (8 h light / 16 h dark) or long day conditions (16 h light / 8 h dark) in growth chambers with 65% humidity. A mixture of Cool White and Gro-Lux Wide Spectrum fluorescent lights with a fluence rate of 125–175 µmol m^{-2} sec^{-1} was used.

For Pi starvation, plants were germinated on MS plates for 7 days, then shifted to plates with full media lacking Pi ((Conn et al. 2013), 0.8% agar, pH 5.7) and grown for 4 more days. *Nicotiana benthamiana* seeds were surface sterilized and vernalized as described above and grown on soil in long day conditions. Mutant alleles *hyl1*-2 (N564863, SALK_064863,), *abh1-753* (N516753, SALK_016753,), *se-3* (N583196, SALK_083196), *ago1-25*, *ago1-27*, *hst-3* (N24278), *miR164a-4* and *hws-1* as well as miRNA mimicry lines MIM156 (N783223), MIM159 (N783226), MIM319 (N783243), MIM164 (N783232), the $Pro_{MIR156c}$::GUS, $Pro_{MIR164a}$::GUS and the miR156 overexpressor-line 35S::miR156b have been described earlier and were obtained either from the Nottingham Arabidopsis Stock Center (NASC) or from the corresponding authors of the respective publications (Rubio-Somoza et al. 2014; Vazquez et al. 2004; Laubinger et al. 2008; Morel et al. 2002; Todesco et al. 2010;

Schwab et al. 2005; Nikovics et al. 2006a; Gonzalez-Carranza et al. 2007; Franco-Zorrilla et al. 2007; Bollman et al. 2003).

For phenotypic analysis of double mutants between *hws-1* and mutant alleles of *AGO1*, *HYL1*, *ABH1, HST* and *SE,* F_2 or F_3 plants were selected phenotypically, then genotyped for homozygosity of the two respective alleles. The oligonucleotides used for genotyping can be found in **Table S1**.

Transgenes

The *HWS* promoter (2460 bp) and the *HWS* genomic- (4190 bp) and coding regions (1236 bp) were PCR-amplified from genomic DNA and cDNA, respectively. They were cloned into pCR8GWTOPO and recombined with ProQuest Two-Hybrid System (Life Technologies) and pGREEN vectors (Hellens et al. 2000). A detailed list of constructs used in this work can be found in the Supplemental **Table S2**, all oligonucleotides used to amplify the *HWS* fragments are listed in **Table S1**.

Transient expression in *Nicotiana benthamiana* after *Agrobacterium*-mediated plant transformations has been described (de Felippes and Weigel 2010).

Mutant Screen and segregation of MIM156 transgene

Plants from a stable miR156 mimicry (MIM156) line in Col-0 background were subjected to ethyl methanesulfonate (EMS) treatment as described (Weigel and Glazebrook 2002). M_2 plants grown in SD conditions were visually inspected for suppression of MIM156 developmental alterations. Candidate plants were crossed to the Ws-0 accession and genomic DNA of 200-300 pooled F_2 plants was extracted using a CTAB protocol. Sequencing libraries (Illumina TruSeq DNA Sample Preparation Kit) were 10-plexed (Illumina adapters Set A) per flow-cell lane and sequenced on a Illumina HiSeq 2000 instrument to obtain at least 10-fold genome coverage. SHOREmap was used to identify SNPs and mapping intervals (Schneeberger et al. 2009).

The MIM156 transgene was removed through outcrossing to the Col-0 accession. Presence or absence of the transgene was deduced from BASTA resistance/sensitivity.

RNA Analysis

Total RNA was isolated from pooled plate-grown whole seedlings 9 days after sowing using TRIZOL reagent (Life Technologies) and DNAseA (Life Technologies) treatment according to manufacturer's instructions. With RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), reverse transcription was performed on 1-2 µg of total RNA. Quantitative RT-PCR on *HWS*, mature miRNAs, miRNA-precursors and miRNA targets was executed with Maxima SYBR Green 2X Master Mix (Thermo Fisher Scientific) on a CFX384 Real-Time PCR system (Bio-Rad), performing technical triplicates on each sample of biological triplicates using *BETA-TUBULIN2* (At5g62690) or *ACTIN2* (At3G18780) as reference genes. Biological replicates are averaged from technical triplicates, horizontal bars show the mean of the biological triplicates. Evaluation and visualization were done with Microsoft Excel for Mac (Version 14.6.6). All oligonucleotides used for RT-PCR experiments are listed in **Supplementary Table 1**.

Histochemical Analysis

Seedlings from at least five independent GUS reporter T_2 lines were inspected 10 days after sowing (DAS). Activity of the GUS reporter was assessed as described (Weigel and Glazebrook 2002), using 20mM potassium-ferro- and 20mM potassium-ferricyanide.

Protein Analyses

T_1 seedlings expressing 35S::GFP-HWS and 35S::GFP-mHWS were BASTA-selected on soil and harvested at 21 days for total protein extraction from three to six whole rosettes as tissue pools. Protein was extracted from ~300 to 1000 mg of ground tissue using equal amounts [w/v] of extraction buffer (50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA; 10% [v/v] glycerol; 1 mM DTT; one tablet of Complete Protease Inhibitor Cocktail per 10 ml buffer). Protein concentration was measured using Bradford solution (Bio-Rad). Expression of the fusion protein was tested by Western blot using GFP-trap (ChromoTek), and appropriate pools were chosen for CoIP with GFP-trap or anti-AGO1 (Agrisera) antibodies.

For protein expression analyses, leaves of *Nicotiana benthamiana* transiently co-transformed with 35S::GFP-HWS or 35S::GFP-mHWS and 35S::AGO1-HA were harvested three days after infiltration. Protein abundance was measured by Western

blot using anti-AGO1 (Agrisera), anti-GFP (Santa Cruz Biotech) or anti-UBQ (Santa Cruz Biotech), equal loading was confirmed using protein staining with either Ponceau red or Coomassie blue.

Interaction experiments in yeast were performed using the ProQuest Two-Hybrid System (Life Technologies) and yeast strain AH109. To reduce autoactivation of some constructs, 5 mM of 3-AT (3-amino-1,2,4-triazole) were added to the selection medium.

Mass spectrometry

Pools of BASTA-selected T_1 seedlings expressing 35S::GFP-HWS and 35S::GFP-mHWS and GFP-overexpressing control plants were frozen in liquid nitrogen and total protein was extracted from up to 1 g finely ground tissue with equal amounts [w/v] of extraction buffer (140 mM NaCl; 8 mM $Na_2HPO_4*7H_2O$; 2 mM KH_2PO_4, pH7.4; 1 mM EDTA; 0.1% [v/v] Triton X-100; 1 tablet of Complete Protease Inhibitor Cocktail (Roche) per 10 ml buffer). Protein concentration was measured using Bradford solution (Bio-Rad) and GFP-expression was verified by Western.

Total protein extracts were purified using GFP-trap metal beads (ChromoTek). A small fraction was resolved on a PAGE gel for staining with the SilverQuest™ Silver Stain Kit (Life Technologies). LC-MS/MS analysis (120 min, Top15HCD) was performed after tryptic in gel digestion, using a Proxeon Easy-nLC (Proxeon Biosystems) coupled to an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) (Borchert et al. 2010). Resulting data was analyzed with MaxQuant v.1.2.2.9 (Cox and Mann 2008; Cox et al. 2011). Spectra were searched against an *Arabidopsis thaliana* database including the protein sequences of the HWS::GFP fusion proteins. Raw data was processed with a setting of 1% for the false discovery rate (FDR).

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We thank Diep Tran for support and suggestions with protein work, Jim Carrington and coworkers for *ago1-25* and *ago1-27*, Jeremy A. Roberts for *hws-1*, Jia-Wei Wang for Pro$_{MIR156c}$::GUS, and Patrick Laufs for Pro$_{MIR164a}$::GUS seeds. We are grateful to Rebecca Schwab, Ignacio Rubio Somoza, and Pablo Manavella for valuable discussion and input for experiments and reviewing the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

M.D.C. and P.L. designed and performed most of the experiments. M.D.C. designed and executed the screen and identified the suppressor. P.L., M.D.C. and D.W. wrote the manuscript.

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FIGURES

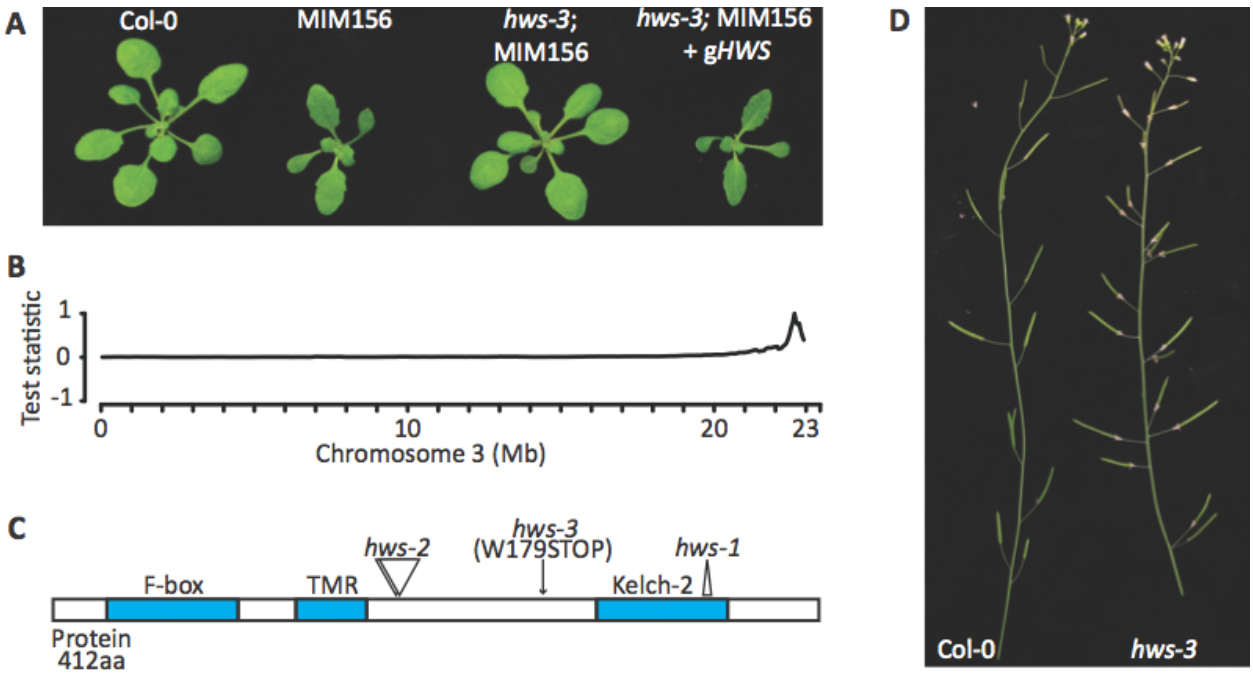


Figure 1. Characterization of the *hws-3* mutant. (*A*) Phenotype of Col-0, MIM156, *hws-3* and *hws-3; gHWS*, the latter two also in the MIM156 background. *(B)* Chromosome 3 SHOREmap results for *hws-3*. *(C)* Location and effects of the mutations on the HWS protein. Annotated or predicted domains are marked in orange. *(D)* Sepal-fusion "skirt" phenotype and phyllotactic distortion in *hws-3* compared to Col-0.

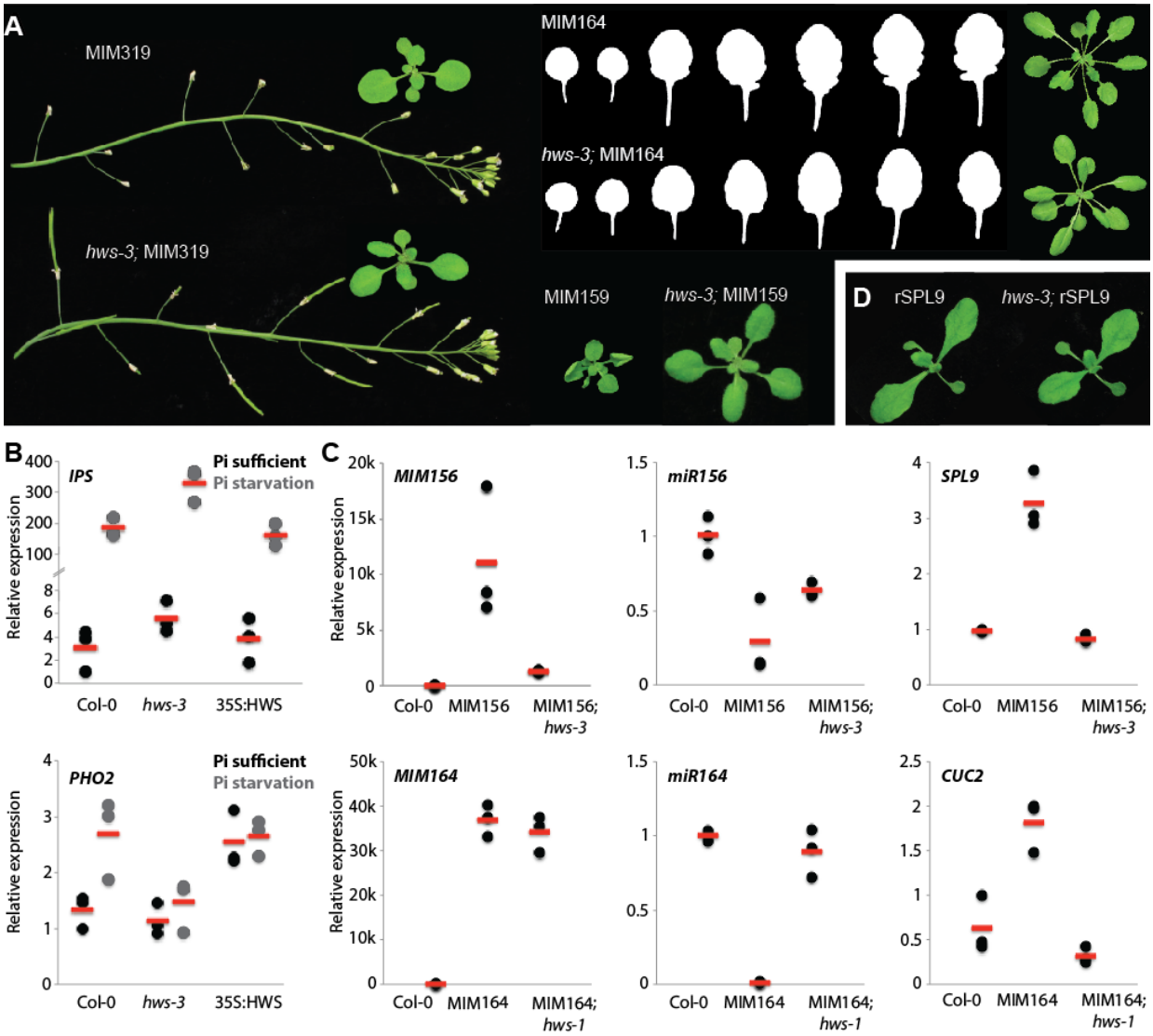


Figure 2. *hws* mutation affects *MIM* transgene and endogenous phenotypes. *(A)* Phenotypic rescue of MIM159, MIM319 and MIM164 in *hws-3* background. *(B)* Relative expression of *IPS* and *PHO2* in Col-0, *hws-3* and *hws-1* plants harboring 35S::HWS. *(C)* Relative expression of *MIM156*, *miR156*, *SPL9* in Col-0, MIM156 and MIM156; *hws-3* and of *MIM164*, *miR164* and *CUC2* in Col-0, MIM164 and MIM164; *hws-1*. *(D)* miR156 resistant rSPL9 wild type and in *hws-3* mutant background.

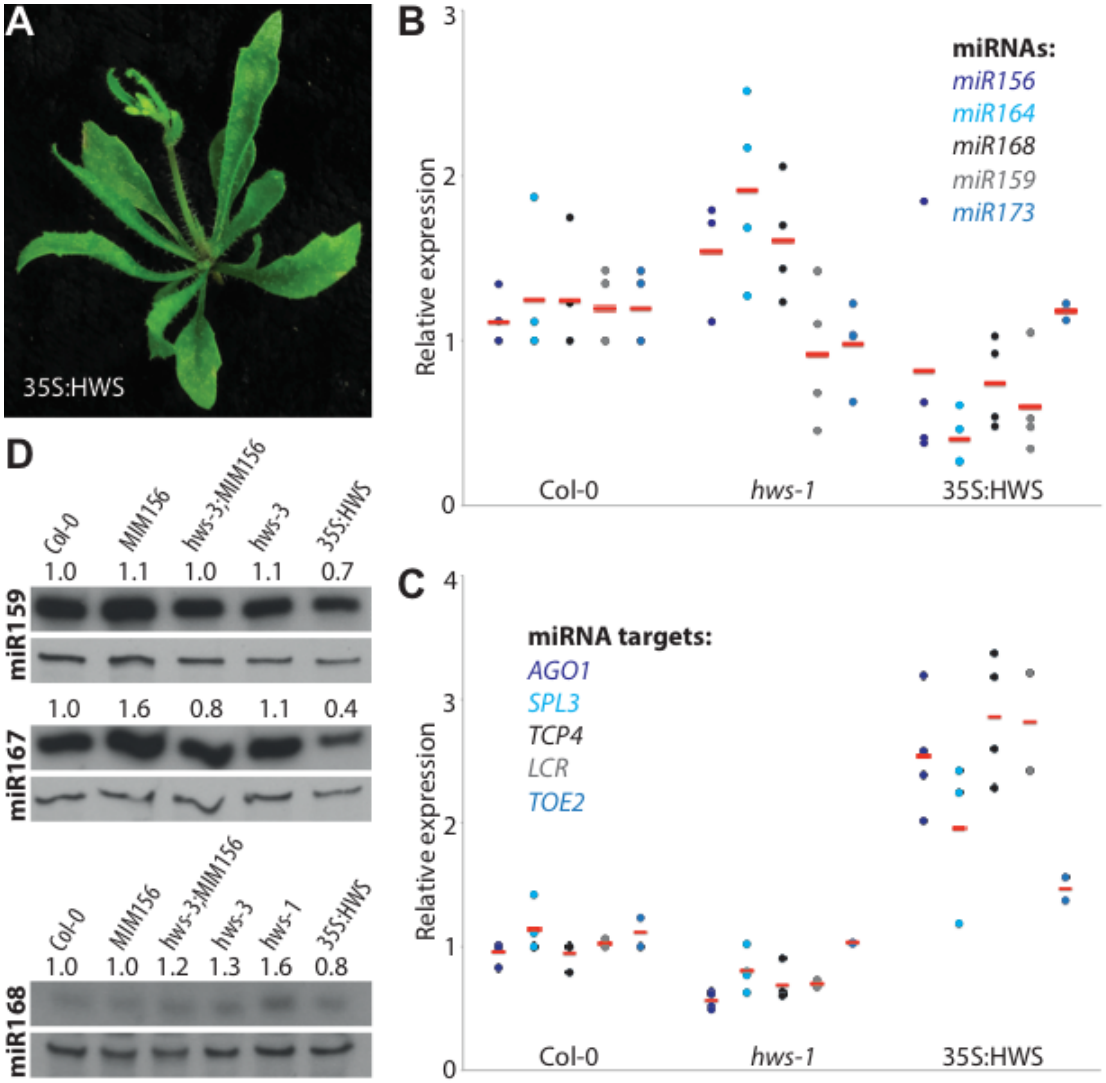


Figure 3. Effects of *hws* and *HWS* overexpression on miRNA and miRNA target steady state levels. *(A)* Hyponastic, serrated leaf phenotype of T_1 35S::HWS plant in the *hws-1* background. (*B* and *C*) Levels of mature miRNAs and miRNA targets in Col-0, "*hws-1* and 35S::HWS as measured by RT-qPCR. Dots represent biological replicates, bars indicate mean of biological replicates. *(D)* Mature miRNA levels in Col-0, *hws-1*, *hws-3*, MIM156, *hws-3*; MIM156 and 35S::HWS as determined by RNA blotting.

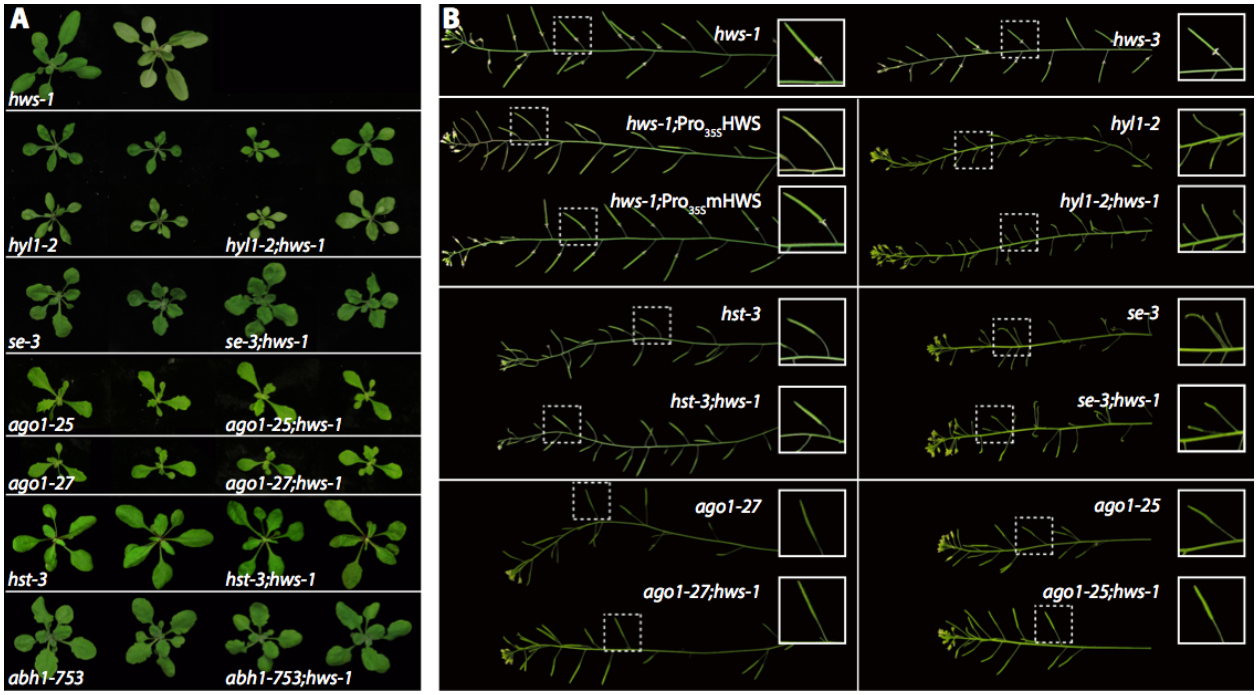


Figure 4. In double mutants of *hws-1* and major miRNA biogenesis factors, abscission is restored, but miRNA-related defects are retained. (*A*) Rosette phenotype of single and double homozygous F_3 plants between *hws-1*, *hyl1-2*, *se-3*, *ago1-25*, *ago1-27*, *hst-3* and *abh1-753* at ~21 DAS. *(B)* Abscission phenotype of double mutants of *hws-1, hws-3*, *hyl1-2*, *hst-3*, *se-3*, *ago1-25*, *ago1-27* as well as T_1 of 35S::HWS and 35S::mHWS in *hws-1* background.

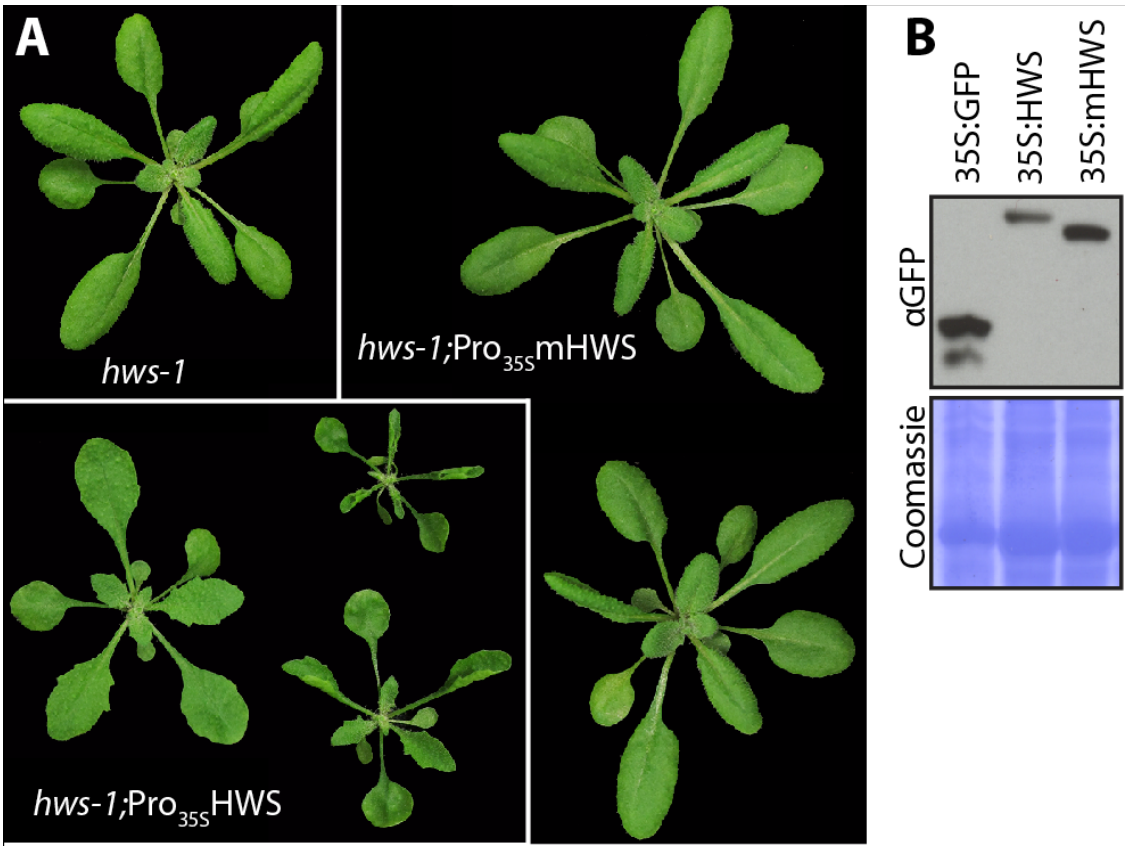


Figure 5. *HWS* overexpression with and without the F-box domain. *(A)* Rosette phenotype comparison of *hws-1* and *hws-1* with 35S::HWS or 35S::mHWS at ~21 DAS. *(B)* Protein levels of HWS-transgenes in transgenic background from *Arabidopsis* T_1 and a stable 35S::GFP line. Coomassie stainings show equal loading.

Supporting Information

Additional supporting information may be found in the online version of this article.

Supplementary Figure 1. Phenotypic characterization of *hws*, 35S::HWS and *hws*; MIM156.

Supplementary Figure 2. Skirt phenotype suppression in MIM159 and MIM319.

Supplementary Figure 3. Phenotypic connection between HWS and miRNA biogenesis.

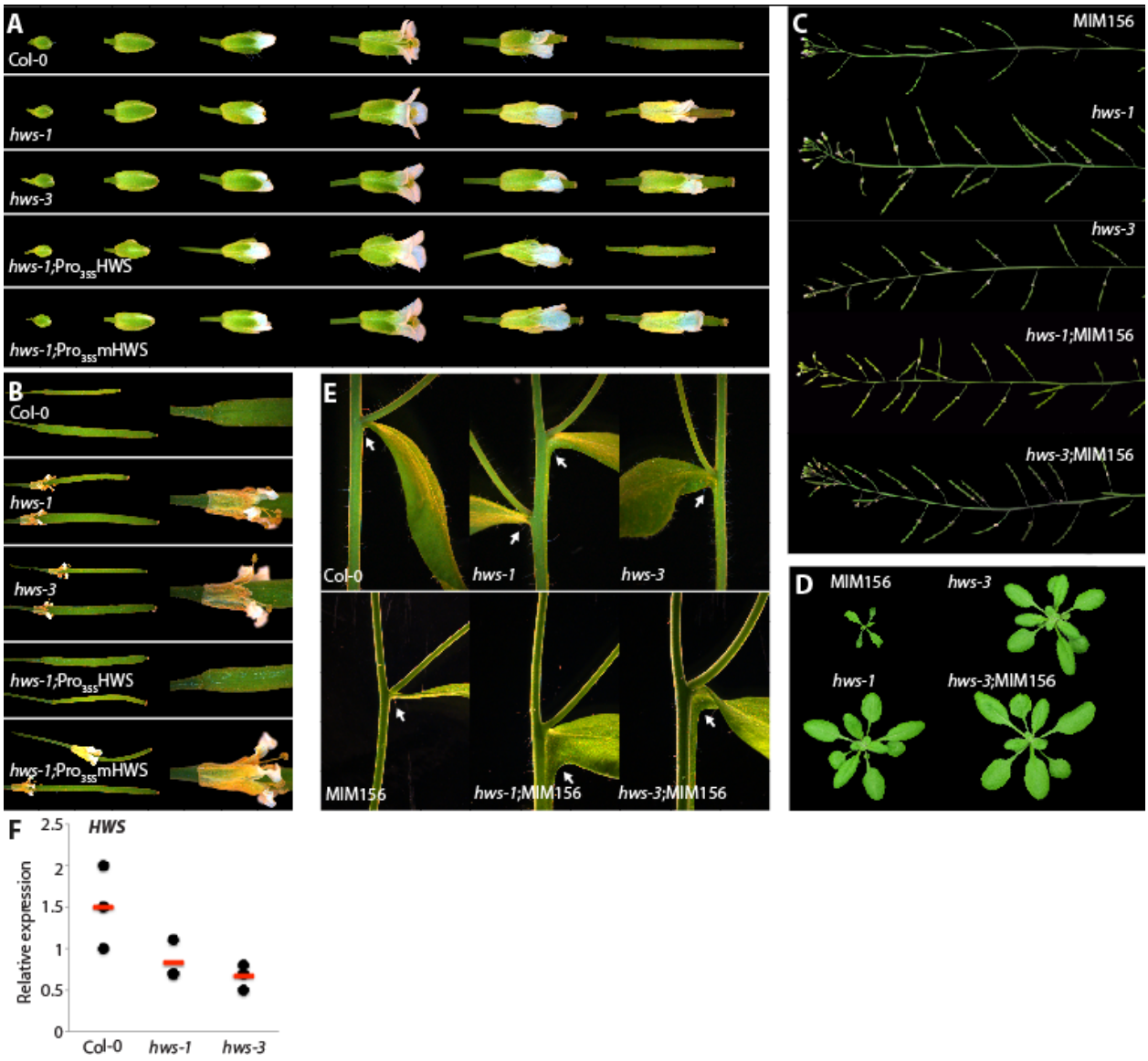
Supplementary Figure 4. Yeast-2-Hybrid interaction screen of HWS with miRNA biogenesis factors.

Supplementary Figure 5. Functional connection between HWS, AGO1 and ubiquitination.

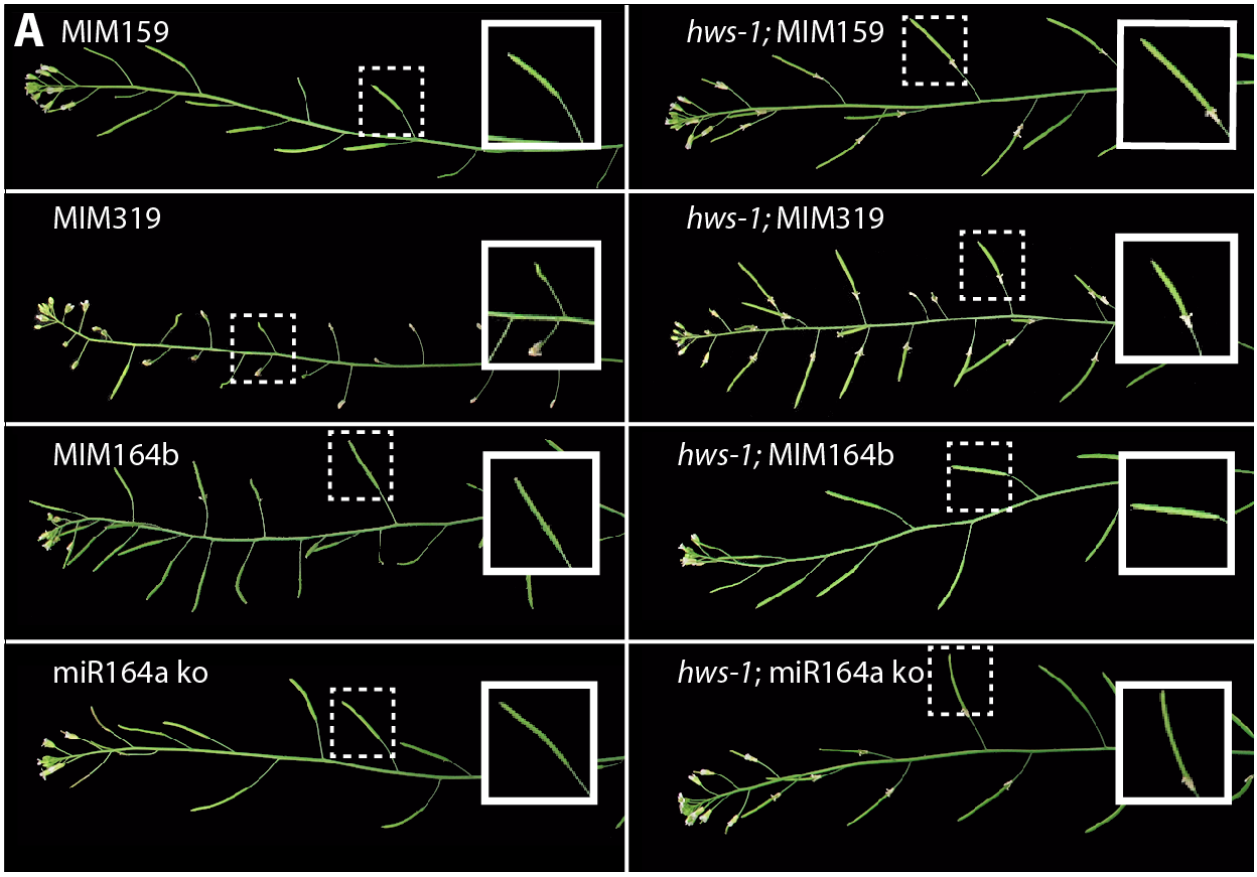
Supplementary Table 1. DNA oligonucleotide primers and probes.

Supplementary Table 2. Plasmids. Constructs for plant transformation are based on pGREEN and confer either Basta or kanamycin resistance in plants.

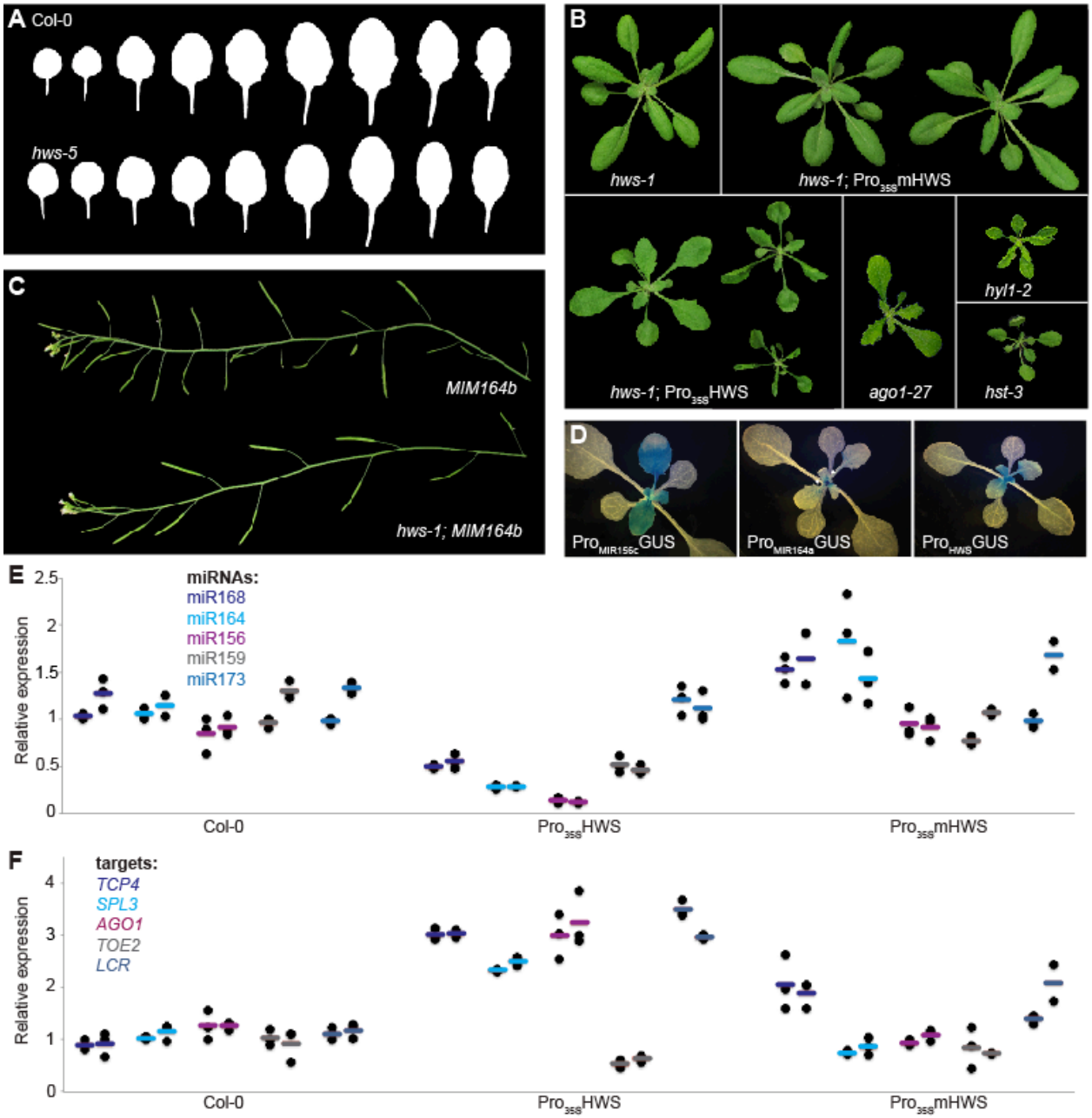
Supplementary Figures



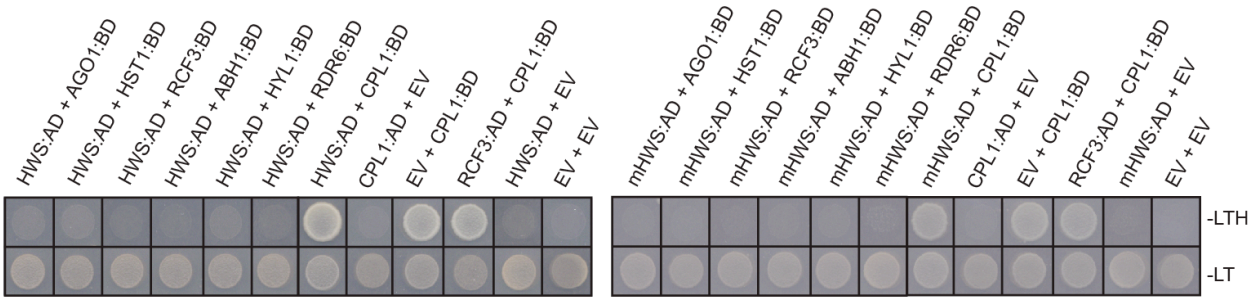
Supplementary Figure 1. Phenotypic characterization of *hws*, 35S::HWS and *hws*; MIM156. *(A)* Flower and *(B)* silique phenotypes of Col-0, *hws-1*, *hws-3*, *hws-1*; 35S::HWS and *hws-1*;35S::mHWS. *(C)* Skirt, *(D)* rosette *(E)* and cauline leaf fusion phenotype and suppression in MIM156, *hws-1*, *hws-3*, *hws-1*; MIM156 and *hws-3*; MIM156. *(F) HWS* expression level as measured by RT-qPCR in Col-0, *hws-1* and *hws-3*.



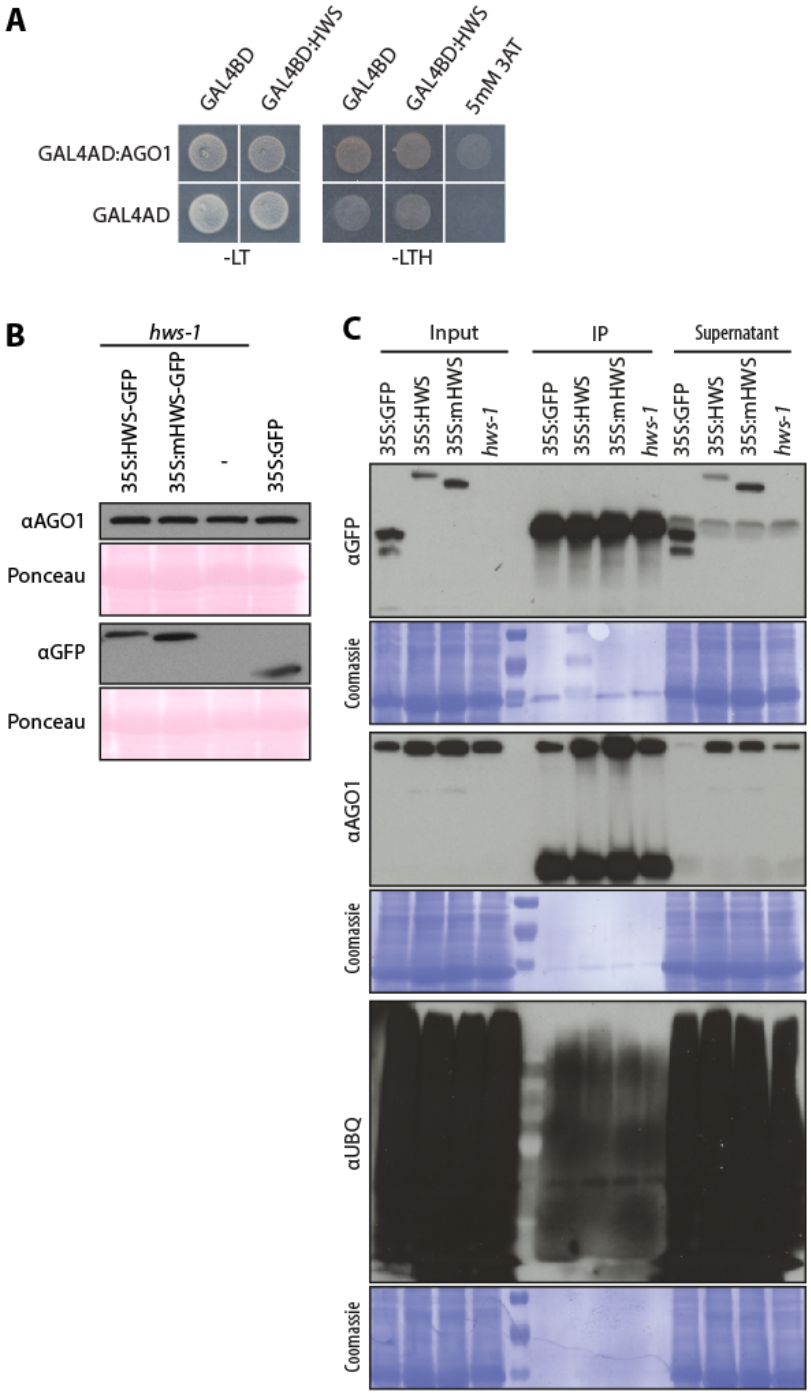
Supplementary Figure 2. Skirt phenotype in MIM159, MIM319, MIM164b and miR164a ko plants with wild-type and *hws-1* background.



Supplementary Figure 3. Phenotypic connection between HWS and miRNA biogenesis. *(A)* Leaf series with serration patterns of Col-0 and *hws-3*. *(B)* Skirt suppression of *hws-1* in MIM164b. *(C)* Rosette phenotype comparison of *hws-1*, *hws-1*; 35S::HWS, *hws-1*; 35S::mHWS, *ago1-27*, *hyl1-2* and *hst-3*. *(D)* Histochemical detection of Pro*MIR156c*::GUS, Pro*MIR164a*::GUS and Pro*HWS*::GUS in rosettes. (*E*) Levels of mature miRNAs in Col-0, *hws-1* and *hws-1*; 35S::mHWS as measured by RT-qPCR. (*F*) Levels of miRNA targets in Col-0, *hws-1* and *hws-1*; 35S::mHWS as measured by RT-qPCR.



Supplementary Figure 4. Yeast-2-Hybrid interaction screen of mHWS with miRNA biogenesis factors.



Supplementary Figure 5. Functional connection between HWS, AGO1 and ubiquitination. *(A)* Yeast-2-Hybrid interaction screen of mHWS with and without the F-box with the AGO1 N-domain. *(B)* AGO1 and GFP-control protein levels in 35S::HWS-GFP, 35S::mHWS-GFP, 35S::GFP and *hws-1*. Ponceau staining indicates equal loading. *(C)* AGO1-IP of 35S::HWS-GFP, 35S::mHWS-GFP, 35S::GFP and *hws-1*. Protein levels of AGO1, GFP and UBQ in input, IP and supernatant fraction. Coomassie staining indicates equal loading.

Supplementary Tables

Supplementary Table 1. Oligonucleotides.

Gene/Allele	Sequence	Purpose
miR159a	TAGAGCTCCCTTCAATCCAAA	RNA blot
miR167	TAGATCATGCTGGCAGCTTCA	RNA blot
miR168	TGGCATACAGGGAGCCAGGCA	RNA blot
U6	GCTAATCTTCTCTGTATCGTTCC	RNA blot
generic miR	GTGCAGGGTCCGAGGT	qPCR
miR156a-f	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC	RT
	GCGGCGGTGACAGAAGAGAGT	qPCR
miR159a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC	RT
	GCGGCGTTTGGATTGAAGGGA	qPCR
miR164a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCACG	RT
	AGGACATGGAGAAGCAGGGCA	qPCR
miR168	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCC	RT
	GGTCGTCGCTTGGTGCAGGTC	qPCR
miR173	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGATT	RT
	AGGACATTCGCTTGCAGAGAGA	qPCR
ACTIN	F:GCCATCCAAGCTGTTCTCTC	qPCR
	R:GCTCGTAGTCAACAGCAACAA	
AGO1	F:TCGGTGGACAGAAGTGGGAATA	qPCR
	R:TCAGCAGTAGAACATGACACG	
CUC2	F:CTCTTACCATTTCATCTCAAG	qPCR
	R:TACCGCTGCTTACGCTCACAG	
HWS	F:CGCGAGTAGTGGCACGGATG	qPCR

	R:GTAGTAACAGGACATTTCTGAG	
IPS1	F: AGCAATGAAGACTGCAGAAGGC	qPCR
	R: ACCGAAGCTTGCCAAAGGATAG	
LCR	F:CTATCCACAGCACACTTTAC	qPCR
	R:CACAGCATTGTAGGTTATCAG	
PHO2	F: CTGATTGGGATAGCTCCAGCGACA	qPCR
	R: AGAAAATGTTCTGTGCCCGTCCA	
SPL3	F:ACGCTTAGCTGGACACAACGAGAGAAG	qPCR
	R:TGGAGAAACAGACAGAGACACAGAGGA	
SPL9	F:CAAGGTTCAGTTGGTGGAGGA	qPCR
	R:TGAAGAAGCTCGCCATGTATTG	
TCP4	F:CAACCGATACAGGAAACGGAG	qPCR
	R:CTGGTATGCGAAAACCCGAAG	
TOE2	F:ATGGAGAACCACATGGCTG	qPCR
	R:ACTGGACTGATCATGCCCTT	
MIM156	F:CTCTAGAAAGCCGAGCAGTGCTATCGAATGGGAAGCTTCG GTTCCCCTCGGAAT	qPCR
	R:GGTACAACCCAAACATAATGAAG	
MIM164	F:TGCTTCTCCAAGCTTCGGTTC	qPCR
	R:GGTACAACCCAAACATAATGAAG	
abh1-753	F:TCAACCTACGAATTCTCTGGG	Genotyping
	R:TCATCTGCTGCCACTACTGTG	
	T-DNA F:ATTTTGCCGATTTCGGAAC	
ago1-25	F:CCATTGCAGGTGATTTGAAAC (+sequencing)	Genotyping
	R:TCCTGAGATGCCACAACCTGA	
ago1-27	F:TCGGTGGACAGAAGTGGGAATA	Genotyping
	R:CAGTAGAACATGACACGCTTCACATTC (+sequencing)	
hws-1	F:TCAAGCGGAATCTTGATGAGGAG	Genotyping
	R:ATCATCCGTGCCACTACTCGC	
hws-3	F:GAGTTACTCAGTCTCGATAGTG	Genotyping

	R:ATCAAGCCGTGGCGATGATC	
hst-3	F:ATGGAAGATAGCAACTCCACGGC	Genotyping
	R:CAAACACCATCATAACAGTGCACCAACT	
hyl1-2	F:TTGCAGGAGTATGCTCAGAAG	Genotyping
	R:AACCATGAGCTTCCCTTTAAACCTTC	
se-3	F:ATGGCCGATGTTAATCTTCC	Genotyping
	R:TTTGAGGATTTCCACTGTTGG	
	T-DNA F: GCGTGGACCGCTTGCTGCAACT	

Supplementary Table 2. Plasmids.

Name	ID	Description
rSPL9	MC004	SPL9 promoter driving N-terminal citrine fused to mutated SPL9 resistant to miR156, fused to the SPL9 3‘ UTR
ProHWS:GUS	MC007	HWS promoter:GUS fusion
GAL4AD:HWs	MC009	GAL4 activation domain fusion to HWS
Pro35S:Citrine-HWS	MC021	Pro35S driving N-terminal Citrine fused to HWS
Pro35S:Citrine-mHWS	MC040	Pro35S driving N-terminal citrine fused to mutated HWS lacking the F-box domain
GAL4AD:mHWS	MC042	GAL4 activation domain fusion to mutated HWS lacking the F-box domain
GAL4BD:mHWS	MC043	GAL4 binding domain fusion to mutated HWS lacking the F-box domain
GAL4AD:RCF3	pPM384	GAL4 activation domain fusion to RCF3
GAL4BD:RCF3	pPM385	GAL4 binding domain fusion to RCF3
GAL4AD:CPL1	pPM358	GAL4 activation domain fusion to CPL1
GAL4BD:CPL1	pPM359	GAL4 binding domain fusion to CPL1
GAL4BD:HYL1	pPM398	GAL4 binding domain fusion to HYL1
GAL4BD:HASTY	pPL031	GAL4 binding domain fusion to HASTY
GAL4BD:ABH1	pPM397	GAL4 binding domain fusion to ABH1
GAL4BD:RDR6	pPM330	GAL4 binding domain fusion to RDR6
GAL4BD:AGO1	pPL026	GAL4 binding domain fusion to AGO1
GAL4AD:AGO1-N	pPL028	GAL4 activation domain fusion to AGO1 N-domain
Pro35S:MIM156	pMT049	Pro35S driving Mimicry156

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